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Picrophilus gen. nov., fam. nov.: a Novel Aerobic, Heterotrophic, Thermoacidophilic Genus and Family Comprising Archaea Capable of Growth around pH 0

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Two species belonging to a novel genus of archaea, designated *Picrophilus oshimae* and *Picrophilus torridus*, have been isolated from two different solfataric locations in northern Japan. One habitat harboring both organisms was a dry, extremely acidic soil (pH < 0.5) that was heated by solfataric gases to about 55°C. In the laboratory both species grew heterotrophically on yeast extract and poorly on tryptone under aerobic conditions at temperatures between 45 and 65°C; they grew optimally at 60°C. The pH optimum was 0.7, but growth occurred even around pH 0. Under optimal conditions, the generation time was about 6 h, yielding densities of up to 10^{10} cells per ml. The cells were surrounded by a highly filigreed regular tetragonal S-layer, and the core lipids of the membrane were mainly bis-phytanyltetraethers. The 16S rRNA sequences of the two species were about 3% different. The complete 16S rRNA sequence of *P. oshimae* was 9.3% different from that of the closest relative, *Thermoplasma acidophilum*. The morphology and physiological properties of the two species characterize *Picrophilus* as a a novel genus that is a member of a novel family within the order *Thermoplasmales*.

Thermoacidophilic microorganisms thrive on coal refuse piles and in the upper oxygenic zones of acidic geothermally heated waters in solfataras. Sulfuric acid arises in these habitats from the oxidation of H_2S, either spontaneously or by the action of sulfur-oxidizing bacteria (1). Except for some heterotrophic species of the bacterial genus *Bacillus*, all organisms isolated from acidic environments with temperatures above 50°C have been archaea of the orders *Sulfolobales* and *Thermoplasmales* (8, 9). Members of the *Sulfolobales* are extreme thermophiles that grow optimally at between 75 and 85°C. With one exception, they are obligate or facultative heterotrophic aerobes and/or chemolithoautotrophs; in the latter case they are capable of gaining energy by oxidation of sulfur compounds to sulfuric acid or by reduction of sulfur to H_2S (20). The first described representative of the order *Thermoplasmales*, *Thermoplasma acidophilum*, was isolated by Darland et al. (2) from a coal refuse pile. It is unique among the archaea in being devoid of a cell envelope. *T. acidophilum* is a facultatively anaerobic heterotroph that requires complex organic extracts for growth. Under anerobic conditions, it grows by sulfur respiration (19, 21). *T. acidophilum* grows optimally at 59°C and was the prokaryotic record-holder in acidophily, with a pH optimum of around 1.8 to 2 and the ability to still, although barely, grow at around pH 0.4 (1, 2). *T. acidophilum* and related acidophiles with similar morphologies and physiological properties that have been combined in the taxon *Thermoplasma volcanium* were later found in various natural habitats around the world (11, 18).

Here we report on the isolation and characterization of members of two species of a novel genus and family of thermoacidophilic archaea, which have been obtained by sampling solfataric environments in northern Japan that resemble habitats of *Thermoplasma* spp. by their moderate temperature (50 to 55°C) and their strong acidity (pH <0.5 to 2.2). These organisms, *Picrophilus oshimae* and *Picrophilus torridus*, have S-layers and clearly outdo *Thermoplasma* spp. in acidophily. A preliminary account of this discovery has been published earlier (17). The genealogy of the novel genus and its nearest relatives is discussed.

MATERIALS AND METHODS

Culture conditions. If not mentioned otherwise, all isolates of *Picrophilus* and *T. acidophilum* were grown in the medium originally described by E. A. Freundt (23) with 0.2% yeast extract as a carbon source. The pH was adjusted with H_2SO_4. Usually 50-ml cultures were incubated with moderate shaking at 60°C. The pH of the medium was measured with an Ingold mettler electrode (type 405-60-57/120) at 20°C. Different dilutions of HCl were used as references for low pH values.

Strictly anaerobic cultures were tested in closed vessels with medium deprived of traces of oxygen by the addition of H_2S-water with resazurin (1 mg/liter) as a redox indicator. The atmosphere consisted of 160 kPa of CO_2 with or without the addition of 40 kPa of H_2. To test for sulfur respiration, 6 g of elemental sulfur per liter was added.

Electron microscopy. Negative staining was done with 1 to 2% uranyl acetate. Specimens were visualized in a Zeiss 109 electron microscope. For freeze-etching, the concentrated cell suspension was frozen between two copper platelets in a cryojet (Balzers Union, Liechtenstein). Freeze-etching was carried out in a freeze-etch unit (BAF 360; Balzers AG) at −100°C for 0.5 min. The fracture faces were shadowed with 2 nm of platinum-carbon at an elevation angle of 45°. The metal film was backed with a 10-nm-thick carbon layer. Replicas were washed with water and kept in 70% sulfuric acid overnight. Replicas were examined in a Philips EM CM12 electron microscope.

DNA-dependent RNAP. Purification of the DNA-dependent RNA polymerase (RNAP) by the polymin-P method as described for other archaea (30) yielded an inactive, incomplete enzyme lacking component A″ and several small components but containing the large subunits B and A′.

Lipid analysis. The lipids were extracted and methanolized as described by De Rosa and Gambacorta (4). The core lipids were analyzed by thin-layer chromatography in comparison with authentic standards from *Sulfolobus solfataricus*, with *n*-hexane–ethylacetate (8:2 for archaeol and 7:3 for caldarchaeol). The polar lipids were purified on a silica gel column as described by De Rosa and Gambacorta (4) and analyzed by thin-layer chromatography with $CHCl_3$-methanol-

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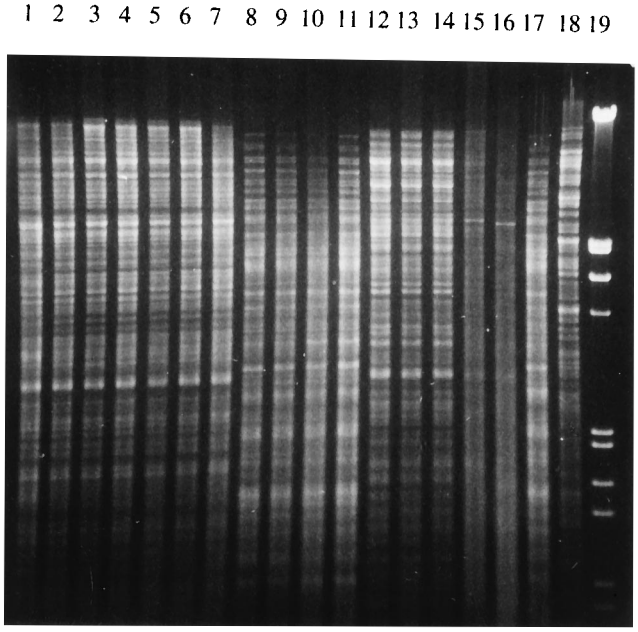

FIG. 1. Agarose gel electropherogram showing *Eco*RI restriction fragment patterns of total DNAs from isolates KAW2/2 (lane 1) and NOB4/1 through NOB4/6 (lanes 2 to 7, respectively), assigned to the species *P. oshimae*; KAW1/1 through KAW1/4 (lanes 8 to 11, respectively), appearing to be identical to *T. acidophilum*; KAW2/3 through KAW2/5 (lanes 12 to 14, respectively), assigned to the species *P. torridus*; and T1/1 and T/2 (lanes 15 and 16, respectively), identical to those in lanes 1 to 7 although not clearly visible in this electropherogram. Lane 17, DNA of *T. acidophilum*; lane 18, DNA of *T. volcanium*; lane 19, size marker (λ DNA cut with *Eco*RI and *Hin*dIII).

H_2O (65:25:4). The spots were visualized by charring with cerium sulfate and by specific reagents for glyco- and phospholipids.

DNA analysis and determination of G+C content. DNA was prepared by CsCl gradient centrifugation as described previously (29). Two micrograms of total DNA was cut with *Eco*RI and separated on a 1% agarose gel. For determination of the G+C content, the DNA was digested with P1 nuclease and the mononucleotides were analyzed by high-pressure liquid chromatography (HPLC) as described previously (31). The DNAs of phage T7 (50 mol% G+C) and of *S. solfataricus* (34 mol% G+C) were used as references.

Cloning and DNA sequence analysis of the 16S rRNA gene. The 16S rRNA gene was amplified by PCR from the chromosomal DNA of isolate KAW2/2 by using the following two primers, which were deduced from the 5′ and 3′ ends, respectively, of aligned 16S rRNA genes of various organisms (underlined): primer 1, 5′-TATA**GCGGCCGC**ATTGYGKTTGAT CCYGSC-3′; primer 2, 5′-ATAT**GCGGCCGC**GGAGGTGATCCAGCCGCAG-3′. Each primer had an extension at its 5′ end that contained a *Not*I restriction site (boldface) for cloning the PCR product. A specific product was obtained by using an annealing step in the PCR at 45°C. After being cut with *Not*I, the product was ligated into pBluescript KS+ (Stratagene) and sequenced from both strands via cycle sequencing with fluorogenic nucleotides (ABI). The same primers were used for amplification of the 16S rRNA gene of isolate KAW2/3. This product was used for direct sequencing with a nested primer derived from the 16S rRNA sequence of isolate KAW2/2.

Determination of phylogenetic position. 16S rRNA sequences were aligned, with consideration of the secondary structure, and positions suitable for phylogenetic inferences were selected as described by Douglas et al. (5). Sequence differences and most-parsimonious trees were inferred with PAUP (26); phylogenetic distances and neighbor-joining trees were inferred with DNADIST and NEIGHBOR (6) as described by Jukes and Cantor (7), considering transversions only. Maximum-likelihood trees were inferred with DNAML (6) and fast DNAML (14).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA gene of *P. oshimae* isolate KAW2/2 has been deposited in the EMBL database under accession number X84901.

RESULTS

Sampling and isolation of *Picrophilus* strains. Samples were taken from a solfataric spring in Noboribetsu (NOB4), from a geothermally heated basin with sulfurous deposits at Kawayu Onsen (KAW1), and from a dry, hot soil (obviously a dried small solfataric field) in the vicinity of Kawayu (KAW2), all in Hokkaido, northern Japan. The original temperature of these samples was around 50 to 60°C, and the pH was 2.2 at NOB4 and KAW1 and even below 0.5 at KAW2. The samples were stored at about pH 3 under anaerobic conditions as described previously (29). For enrichment, 50-ml portions of growth medium (see Materials and Methods) containing yeast extract (0.1%) and glucose (1%) as carbon sources

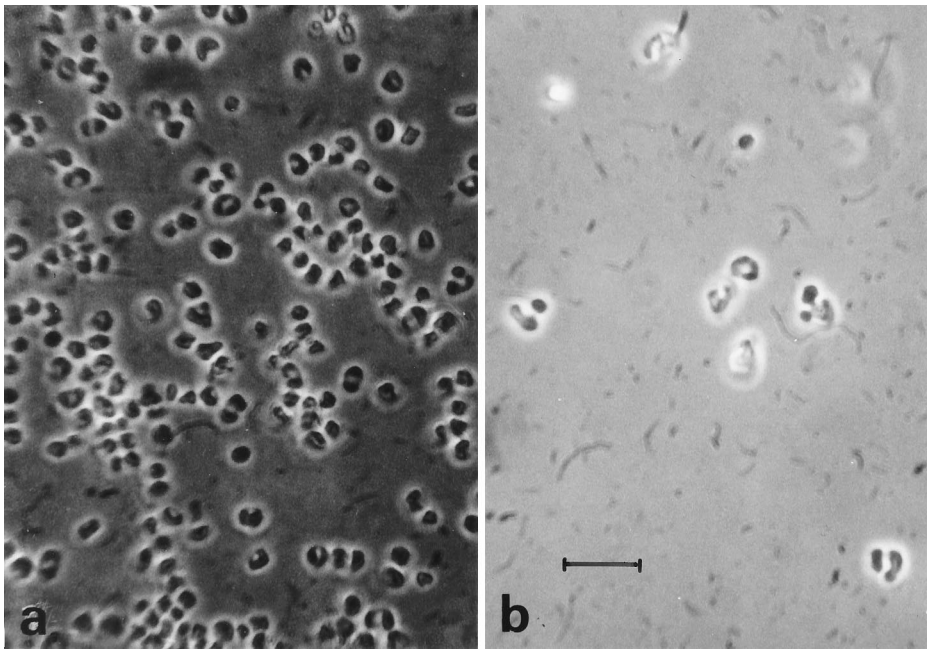

FIG. 2. Phase-contrast micrographs of *P. oshimae* from an early-logarithmic-phase culture (a) and from a late-logarithmic-phase culture (b). Bar, 10 μm.

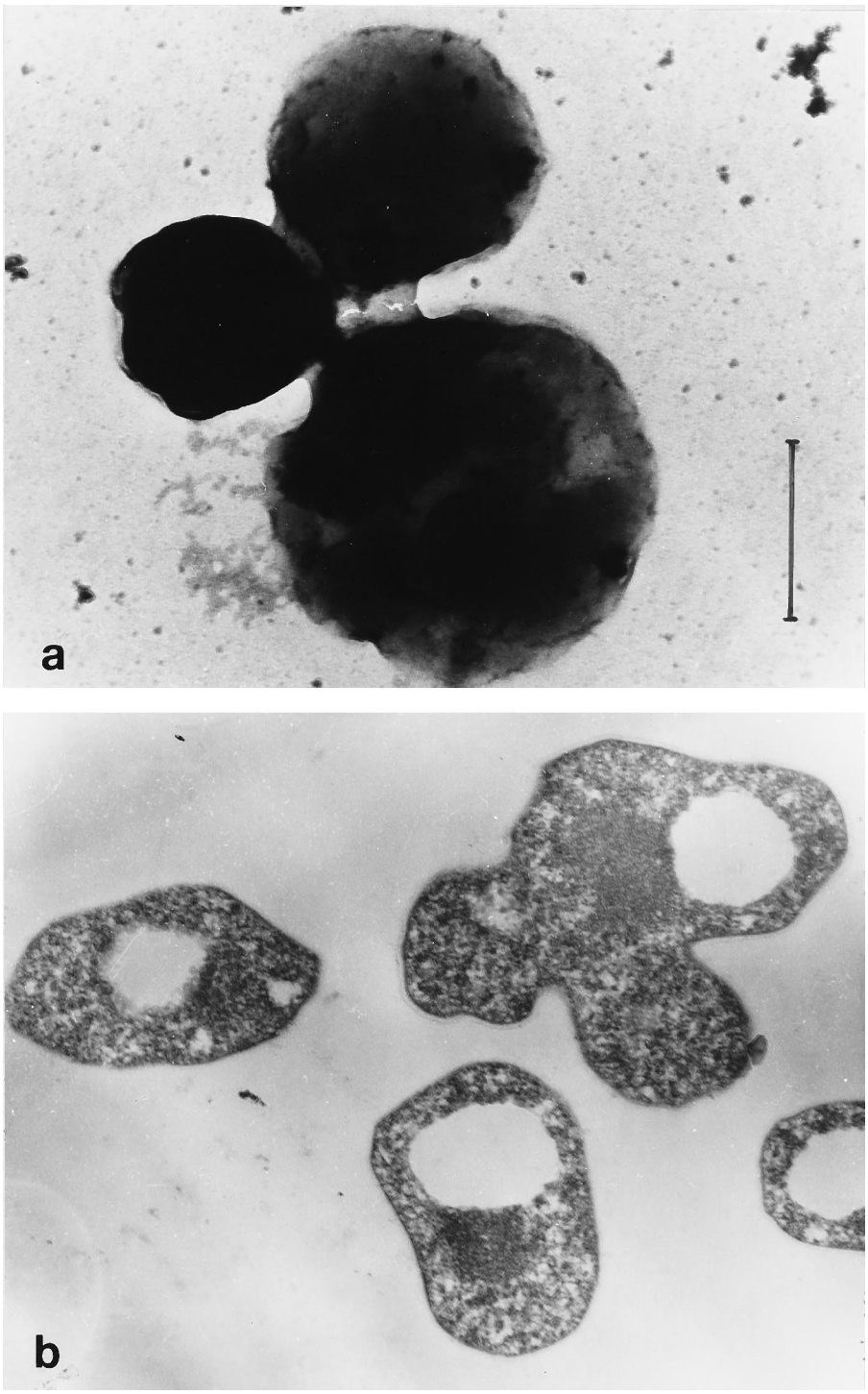

FIG. 3. Electron micrographs of *P. oshimae*. (a) Cells from a logarithmic culture, triplex form, rotary shadowed with Pt; (b) thin section exhibiting an S-layer and cavities, embedded in Epon 812, contrasted with lead citrate, uranyl acetate, and lead citrate. Bar corresponds to 1 μm.

were inoculated with 1 ml each of the samples and incubated aerobically under moderate shaking at 60°C. Clones were isolated from single colonies grown for 6 days on 12.5% starch plates containing the same medium at pH 1. Under these conditions, the plating efficiency approached 100%, and whitish-yellow, convex, shiny colonies 2 to 5 mm in diameter were obtained. We analyzed six clones from NOB4, five clones from KAW1, and five clones from KAW2, each designated by the source and the clone number, and also two clones obtained by plating a culture of *T. acidophilum* kept in the laboratory and originally obtained from E. A. Freundt, Aarhus, Denmark.

In order to distinguish different isolates, we prepared total DNA and compared the *Eco*RI restriction fragment patterns

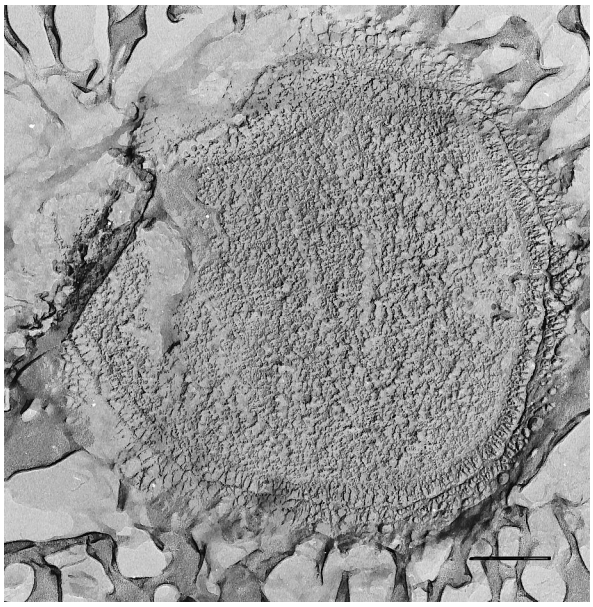

FIG. 4. Freeze-etched cells of *P. oshimae*, showing the S-layer on top of the cytoplasmic membrane and a brush-like structure on the outside. Bar, 210 nm.

obtained by agarose gel electrophoresis (Fig. 1). The patterns of isolates KAW2/2 and NOB4/1 to NOB4/6 (Fig. 1, lanes 1 to 7) appeared to be identical, leading us to assign them to one species, *P. oshimae* (DSM 9789). A similar but clearly different pattern was obtained from KAW2/1 and from KAW2/3 to KAW2/5 (Fig. 1, lanes 12 to 14), and these isolates were assigned to another species, *P. torridus* (DSM 9790). The isolates KAW1/1 to KAW1/4 (Fig. 1, lanes 8 to 11) yielded patterns identical to that of *T. acidophilum* (lane 17). Two clones obtained by plating an "authentic" *T. acidophilum* culture, in contrast, proved to be additional strains of *P. oshimae*, T1/1 and T1/2 (Fig. 1, lanes 15 and 16). The inoculum from which this culture was derived must therefore have been contaminated by *Picrophilus* spp.

If not otherwise stated, the following experiments were carried out with strain KAW2/2 (*P. oshimae*), which was the first obtained in pure culture.

Morphology. The cells of all isolates were irregular cocci of around 1 to 1.5 μm in diameter, resembling *Thermococcus* and *Pyrococcus* cells rather than the irregularly angular cells of *Sulfolobus*, *Pyrodictium*, or *Hyperthermus* spp. (Fig. 2 and 3). In exponentially growing cultures, many duplex and sometimes triplex forms, reminiscent of intermediates of cell division of *Thermococcus* spp., were observed (Fig. 2b and 3b) (28). In electron micrographs of thin sections, large cavities appearing as zones of low electron density were found in the cells, resembling vacuoles but apparently not separated from the cytoplasm by a membrane (Fig. 3).

The cells showed an envelope in the form of an S-layer situated on top of the cytoplasmic membrane (Fig. 3 and 4). It consisted of an outer dense and an inner, almost empty stratum, with the latter structured by thin, widely spaced pillars obviously anchoring the surface layer in the membrane. The height of the whole S-layer of isolate KAW2/2 was approximately 40 nm. The isolated S-layer had tetragonal symmetry and a center-to-center distance of about 20 nm (Fig. 5). Its basic architecture in projection, as seen in the correlation average (Fig. 5, inset) was highly regular and remarkably filigreed. The upper stratum appeared to be denser than that in the S-layer of *Desulfurococcus mobilis* (27), which is also highly regular and with tetragonal symmetry. After freeze-etching, the distinct regular pattern of p4 symmetry was visible on the surface of the cells (data not shown). Fracture faces through the cytoplasm showed an additional brush-like structure on top of the S-layer, which possibly was made up of long polysaccharide chains (Fig. 4). Pili or flagella have not been observed.

Metabolic properties. The organism grew heterotrophically under aerobic conditions on 0.2% yeast extract, yielding cell densities of around 5×10^8/ml with a generation time of 8 h. The addition of sugars (1% glucose, sucrose, or lactose) to the medium resulted in slightly slower growth, but the cultures reached higher cell densities (around 10^9/ml) and had an extended viability (Fig. 6). Therefore, 1% glucose was added to the yeast extract as an additional carbon source for continuous cultivations. No growth occurred on starch, glucose, sucrose, or lactose alone, on Casamino Acids, or when these carbon sources were supplemented with vitamins. On tryptone, poor growth to low final densities occurred. Adding yeast extract to the tryptone medium did not restore the growth rate observed with yeast extract alone, indicating that tryptone compounds inhibited growth.

Growth on 0.3 and 0.5% yeast extract led to final cell densities higher than those obtained with 0.2% yeast extract, although this was preceded by a prolonged lag phase. The addition of 1% yeast extract was even inhibiting. In large-scale cultures, very high cell densities (around 10^{10}/ml) were obtained when yeast extract was added in batches of 20 g/liter each whenever the growth rate started to decrease. Small-scale cultures were aerated by shaking; large-scale cultures required strong bubbling by air to which 5 to 10% CO_2 was added. In the latter case, the doubling time was as low as 6 h.

Growth was inhibited by the addition of 0.2 M sodium chloride to the medium. No growth was observed under aerobic conditions with CO_2 as the sole carbon source and sulfur as the energy source. Under anaerobic conditions on yeast extract with and without sulfur and CO_2 or under conditions for chemolithoautotrophic growth with sulfur and H_2 as the energy source and CO_2 as the carbon source, no growth was observed.

Temperature and pH dependence of growth. *Picrophilus* spp. grew between 45 and 65°C, with an optimum at 60°C (Fig. 7). No growth occurred at 40 and 67°C.

The organisms grew only below pH 3.5. With 0.2% yeast extract at 60°C, the optimal pH was 0.7 (Fig. 8). Even at a pH around 0, significant growth was observed. The growth curve shown in Fig. 9 for pH 0.03 is that of a culture in which 0.9 M H_2SO_4 had been added to the medium. The inocula used for these experiments were from late-logarithmic-phase cultures grown under optimal conditions. By using "adapted" inocula from a culture of *P. torridus* KAW2/3 grown at pH 0.1, significant growth was observed even upon addition of 1.2 M sulfuric acid. The pH in this medium was measured to be -0.06 ± 0.01.

The minimal pH allowing growth of *T. acidophilum* under these conditions was around 0.4, i.e., in accordance with earlier reports (1, 2).

Storage. Cells of *Picrophilus* lysed at above pH 5. In order to obtain viable glycerol stocks, the cells were suspended in a basal salt medium of pH 4.5 containing 20% glycerol and kept at −70°C.

Sensitivity to antibiotics. Table 1 shows the MICs of the antibiotics tested. As expected, those drugs that interfere with bacterial cell wall synthesis had no effect. However, significant inhibition was observed with chloramphenicol, novobiocin, and rifampin. All three drugs have been shown to inhibit growth of

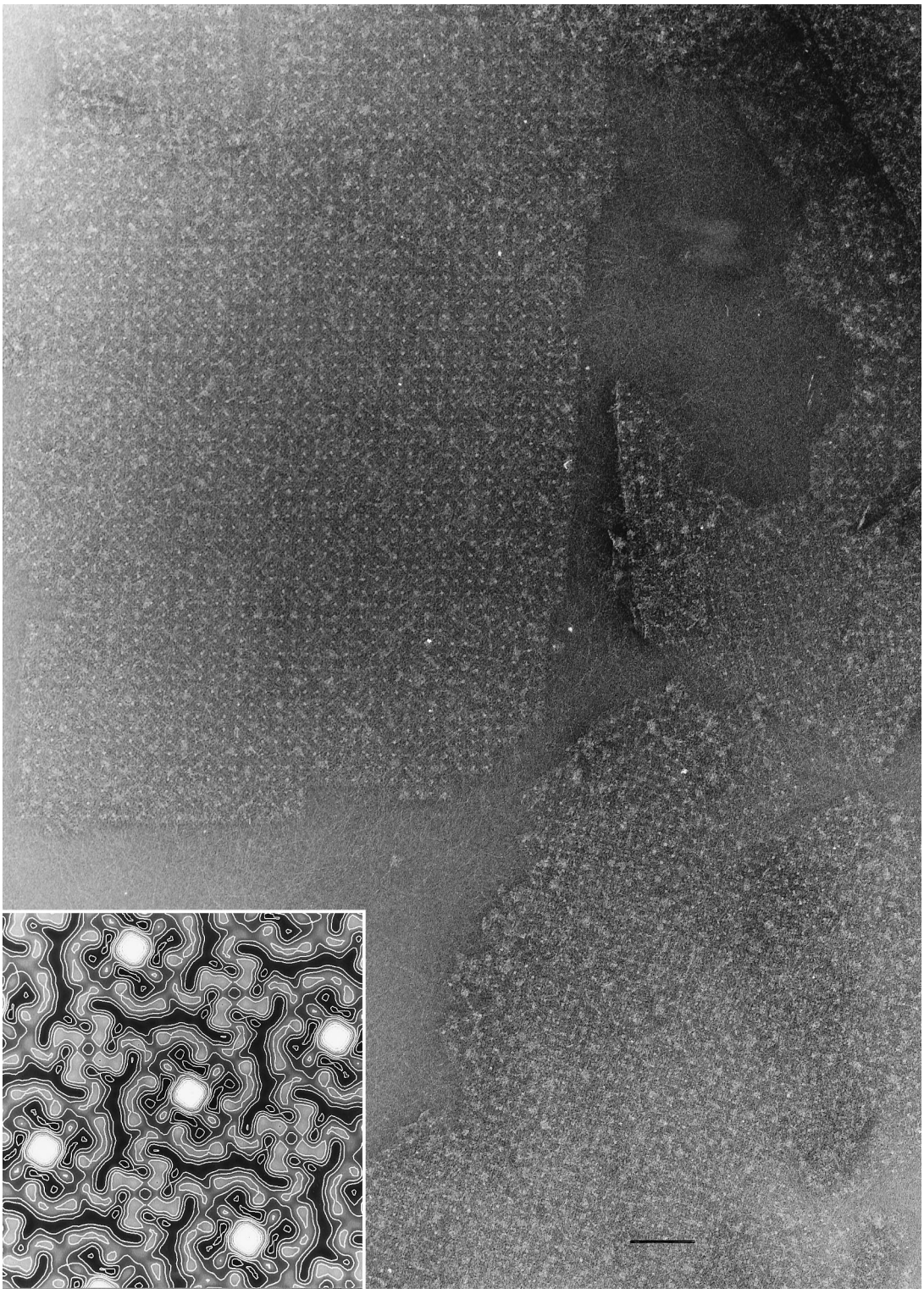

FIG. 5. Electron micrograph of a surface layer fragment from *P. oshimae*, negatively stained with uranyl acetate. Bar, 100 nm. The inset shows the correlation average.

some other archaea as well, although the mode of inhibition must be different from that in bacteria in the case of rifampin (unpublished data from our laboratory).

DNA-dependent RNAP. Purification of the RNAP of *P. oshimae* by the polymin P method, as described for other thermophilic archaea (30), yielded an inactive, incomplete enzyme. As shown by Western blotting (immunoblotting), subunits A″ and E and some unidentified small components were absent. In the immunodiffusion assay of Ouchterlony (15), no cross-reactions between the RNAPs of *P. oshimae*, *T. acidophilum*,

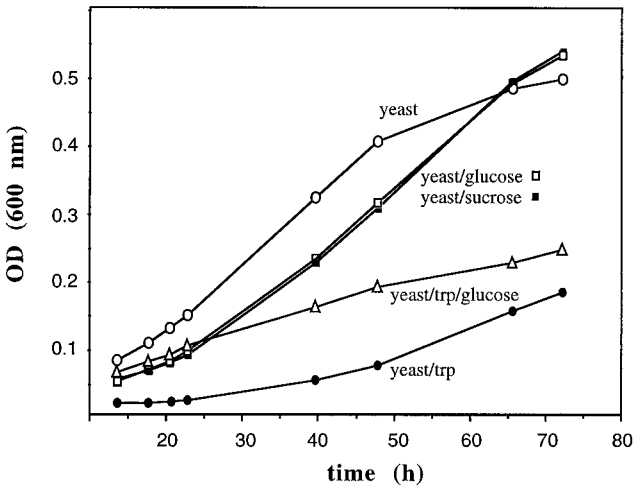

FIG. 6. Growth curves of *P. oshimae* with different carbon sources. Yeast extract (yeast) or tryptone (trp) (0.1%) and glucose or sucrose (1%) were added to the medium. OD, optical density.

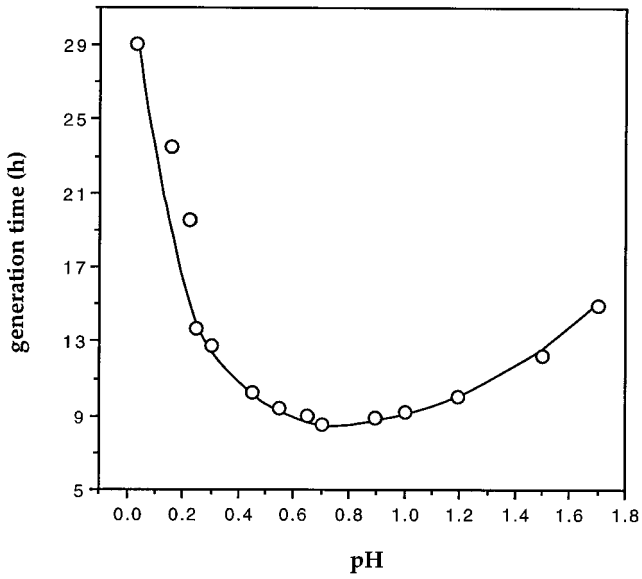

FIG. 8. Optimal pH for growth of *P. oshimae*. Doubling times were calculated from the slopes of the growth curves (not shown).

and *Sulfolobus acidocaldarius* were observed when polyclonal antisera directed against the RNAPs of the latter two organisms were used (not shown).

Lipids. The major core lipid obtained after methanolysis of the polar lipids of *P. oshimae* was caldarchaeol, containing 0+0, 0+1, 1+1, 1+2, 2+2, and 3+2 cyclopentane rings, with mainly 0+0 and 1+2 rings. A trace of archaeol was also found. The abundant polar lipids were a glycolipid (R_f, 0.93), identified as a β-glucosyl derivative of caldarchaeol, and a phosphoglycolipid (R_f, 0.35) in $CHCl_3$-methanol-H_2O (65:25:4), with the latter containing the same sugar moiety, although the phospho-head has not yet been identified.

Plasmids. CsCl gradient centrifugation of the total DNAs of isolates KAW2/2, NOB4/1 to NOB4/6, T1/1, and T1/2 in the presence of 1 mg of ethidium bromide per ml yielded covalently closed circular DNA bands in addition to the chromosomal DNA bands. The *Eco*RI restriction patterns of the plasmid fractions from NOB4/1 to NOB4/6 and T1/1 indicated the presence of a plasmid of about 8.3 kb. That of T1/2 indicated a plasmid of about 8.8 kb, and KAW2/2 harbored both of these plasmids, which showed strong cross-hybridization in Southern analysis (not shown). The *P. torridus* strain KAW2/3 and the *T. acidophilum* isolate KAW1/1 did not contain plasmids.

Phylogenetic position. The G+C content of the DNA of *P. oshimae* isolate KAW2/2, determined by HPLC of the nucleotides, was around 36 mol%.

The 16S rRNA gene of this isolate was amplified via PCR from the chromosome and was cloned into an *Escherichia coli* vector. The complete nucleotide sequence of this gene was determined. The sequence has the highest degree of similarity to that of *T. acidophilum*, with 9.3% deviation. The 16S rRNA

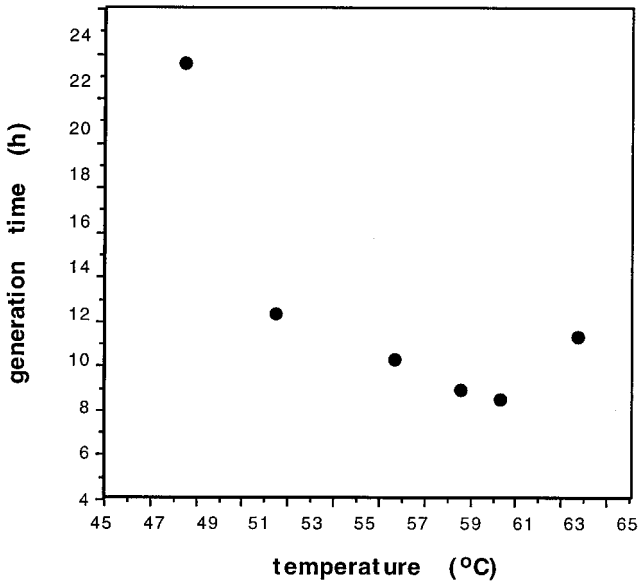

FIG. 7. Optimal growth temperature of *P. oshimae*. Doubling times were calculated from the slopes of the growth curves (not shown).

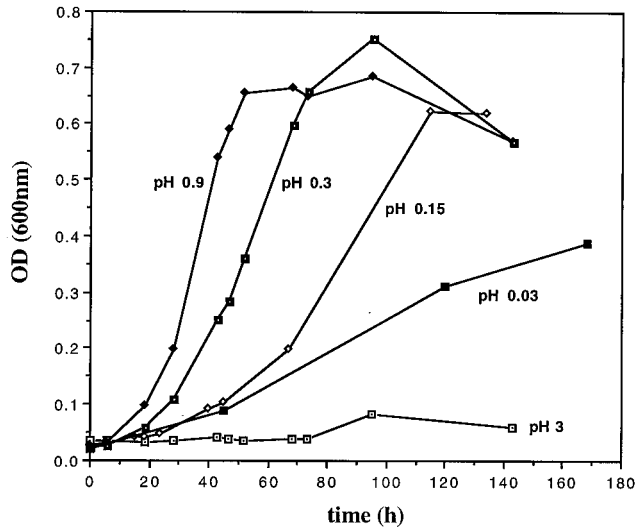

FIG. 9. Growth curves of *P. oshimae* at various pH values. OD, optical density.

TABLE 1. Antibiotic sensitivities[a]

Antibiotic	MIC (μg/ml)
Rifampin	15
Chloramphenicol	40
Streptomycin	100
Novobiocin	<10
Bacitracin	>200
Vancomycin	>200

[a] Cells were grown without antibiotic for one generation before the drug was added.

gene of strain KAW2/3, representing *P. torridus*, was also obtained by PCR amplification, and 250 bp of the 3′-terminal sequence was determined. This region was 3% different from the corresponding section of *P. oshimae*, whereas the deviation between these sequence sections in *P. oshimae* and *T. acidophilum* was 11.5%.

The upper right half of Fig. 10 shows the pairwise sequence differences of 1,404 well-aligned positions of the 16S rRNA genes of representative archaea, including *P. oshimae*, and some of the genes cloned by DeLong and coworkers (3) directly from DNA isolated from natural environments. The latter sequences were also said to be closely related to that of the *Thermoplasma* 16S rRNA gene (3, 12, 14). The lower left half of Fig. 10 shows the pairwise phylogenetic distances derived from a fraction of 994 positions of the aligned sequences inferred with the DNADIST program (6), as described by Jukes and Cantor (7), considering transversions only. These sequences showed a significantly higher degree of conservation than the excluded sequence fraction. They correspond to the conserved sequence fraction selected by Douglas et al. (5) for an 18S rRNA phylogeny.

The closest neighbor of *P. oshimae* and *P. torridus* is *T. acidophilum*. The previously described close relationship between the marine DNA clones and members of the *Thermoplasmales* (3) appears not to be supported by the phylogenetic distances. The average pairwise distance between the oceanic DNA clones and members of the orders *Methanosarcinales* and *Methanomicrobiales*, their closest neighbors in the phylogenetic tree shown in Fig. 11, is apparently less than the distance between these clones and members of the *Thermoplasmales.* Also, the average pairwise distances between members of *Thermoplasmales* and members of the order *Thermococcales*, *Archaeoglobus fulgidus*, and all orders of methanogens (considering not only the sequences included in Fig. 10 but all sequences used for the inference of the tree shown in Fig. 11) are less than phylogenetic distances between members of the *Thermoplasmales* and the oceanic DNA clones.

DISCUSSION

The isolates described here represent two novel species of a genus of thermophilic, heterotrophic, aerobic archaea that grow under extremely acidic conditions. We have therefore termed the genus *Picrophilus* (acid-lover). To our knowledge, *Picrophilus* spp. surpass all hyperacidophilic prokaryotes known so far, i.e., eubacteria of the genera *Thiobacillus* and *Bacillus* and the archaeon *Thermoplasma* sp., in their ability to cope with acidic environments; they grow well even around pH 0 and optimally at pH 0.7. Only a few eukaryotic fungi and an alga have also been reported to be capable of growth at pH values around 0 (1, 22, 24).

The two species of *Picrophilus* have similar physiological properties and are morphologically indistinguishable, but they are clearly distinct in their DNA restriction fragment patterns and in their 16S rRNA gene sequences. *P. oshimae* (named after Tairo Oshima, who helped us organize our sampling trip to northern Japan) represents the type species. The organism was isolated from a hot spring in Noboribetsu, Japan, and from a rather dry, hot soil in Kawayu, Japan. The second species, *P. torridus* (*torridus*, dried, burnt), was isolated only from the latter place, a small, dried-out solfataric field that was still heated by solfataric gases, concentrating the geochemically

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *Saccharomyces cerevisiae*	X	0.384	0.380	0.377	0.399	0.395	**0.410**	0.427	0.410	0.396	0.389	0.396	0.410	0.407	0.408	0.410	0.432
2 *Sulfolobus acidocaldarius*	0.220	X	0.105	0.145	0.195	0.266	**0.267**	0.305	0.298	0.208	0.225	0.228	0.266	0.262	0.267	0.262	0.297
3 *Desulfurococcus mobilis*	0.222	0.037	X	0.117	0.159	0.264	**0.271**	0.280	0.278	0.178	0.206	0.208	0.252	0.244	0.254	0.256	0.269
4 *Thermoproteus tenax*	0.206	0.056	0.044	X	0.170	0.275	**0.284**	0.312	0.310	0.193	0.234	0.227	0.259	0.254	0.271	0.273	0.276
5 *Thermococcus celer*	0.242	0.094	0.074	0.081	X	0.212	**0.217**	0.245	0.243	0.127	0.175	0.171	0.208	0.204	0.211	0.216	0.257
6 *Thermoplasma acidophilum*	0.233	0.113	0.103	0.110	0.072	X	**0.083**	0.244	0.231	0.223	0.230	0.230	0.238	0.251	0.245	0.247	0.327
7 ***Picrophilus oshimae***	**0.240**	**0.121**	**0.109**	**0.116**	**0.073**	**0.013**	X	**0.249**	**0.234**	**0.228**	**0.236**	**0.228**	**0.253**	**0.249**	**0.258**	**0.262**	**0.329**
8 oceanic DNA clone WHARN	0.295	0.163	0.140	0.146	0.107	0.102	**0.102**	X	0.130	0.262	0.257	0.240	0.262	0.259	0.265	0.272	0.353
9 oceanic DNA clone OARB	0.266	0.139	0.121	0.133	0.096	0.099	**0.099**	0.062	X	0.254	0.259	0.215	0.230	0.253	0.260	0.269	0.352
10 *Archaeoglobus fulgidus*	0.239	0.103	0.081	0.085	0.054	0.074	**0.075**	0.106	0.090	X	0.188	0.189	0.186	0.193	0.222	0.231	0.279
11 *Methanococcus vannielii*	0.233	0.106	0.101	0.106	0.065	0.085	**0.084**	0.116	0.107	0.061	X	0.179	0.230	0.225	0.238	0.234	0.311
12 *Methanobacterium formicicum*	0.257	0.105	0.095	0.093	0.064	0.089	**0.087**	0.113	0.088	0.053	0.055	X	0.210	0.194	0.207	0.219	0.301
13 *Methanolobus tindarius*	0.264	0.116	0.100	0.107	0.084	0.085	**0.087**	0.102	0.078	0.073	0.081	0.080	X	0.171	0.227	0.230	0.332
14 *Methanospirillum hungatei*	0.262	0.108	0.094	0.101	0.052	0.083	**0.080**	0.092	0.094	0.052	0.061	0.061	0.049	X	0.201	0.204	0.322
15 *Halobacterium halobium*	0.267	0.113	0.113	0.120	0.085	0.094	**0.098**	0.131	0.119	0.083	0.083	0.071	0.087	0.072	X	0.108	0.326
16 *Halococcus morrhuae*	0.276	0.122	0.120	0.129	0.087	0.106	**0.109**	0.144	0.121	0.087	0.078	0.073	0.095	0.076	0.023	X	0.323
17 *Thermotoga maritima*	0.296	0.199	0.176	0.189	0.174	0.190	**0.186**	0.270	0.260	0.190	0.184	0.189	0.202	0.175	0.199	0.199	X

FIG. 10. Pairwise sequence differences of 1,404 well-aligned positions of the 16S rRNA genes (upper right half) and pairwise phylogenetic distances derived from a fraction of 994 highly conserved positions (lower left half).

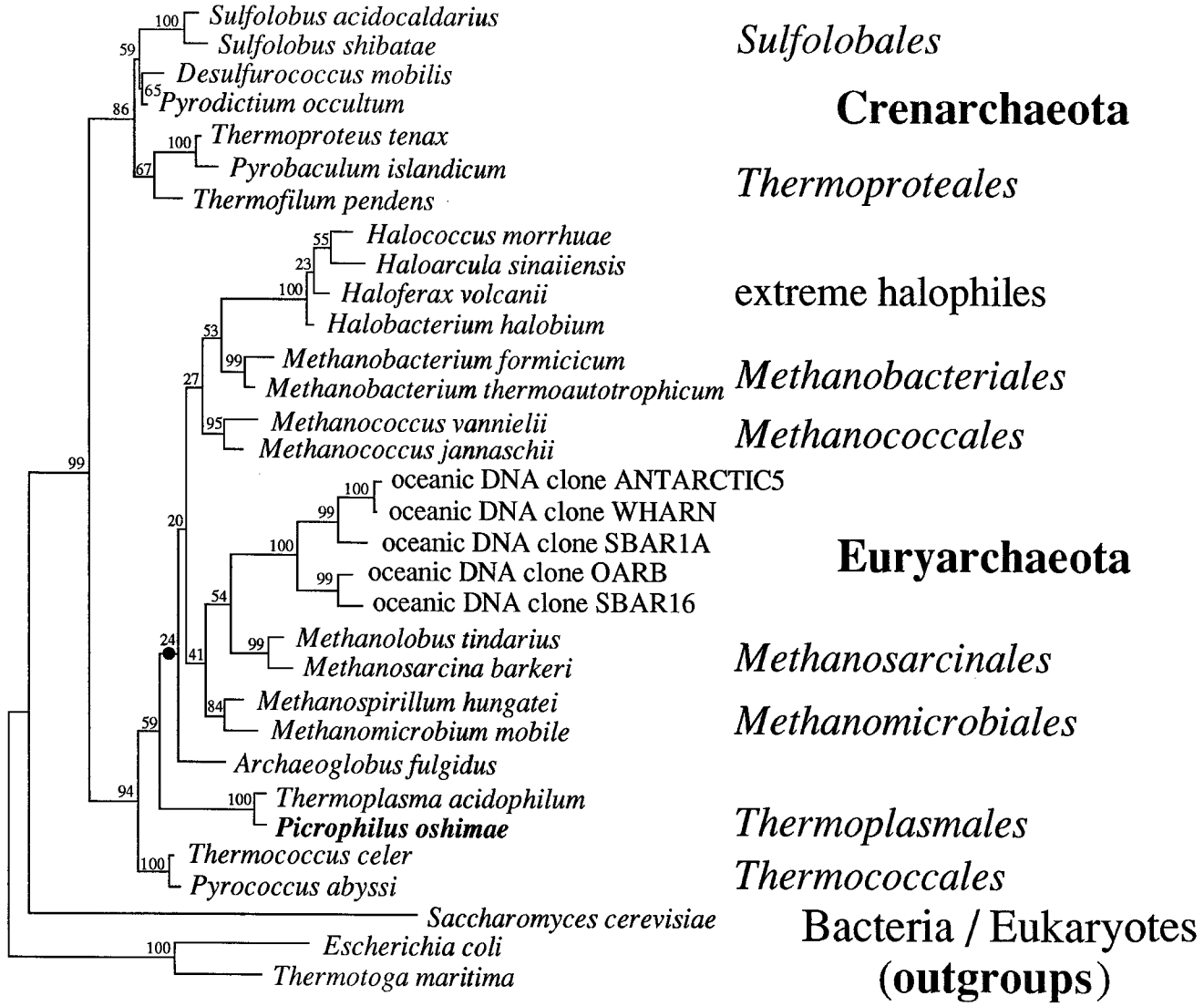

FIG. 11. Tree topology and bootstrap values inferred by using PAUP (26). The lengths of the branches were inferred by DNAML (6). The solid circle shows the phylogenetic location of the split in the *rpoB* gene, encoding subunit B of the DNA-dependent RNAP.

produced or biogenic sulfuric acid. This is a situation frequently encountered in solfataric fields. *Picrophilus* spp. might not have been identified before because such moderately hot sources (around 50 to 60°C) appeared to be less attractive for sampling. Furthermore, enrichment for thermoacidophilic archaea from solfataras has often been performed under anaerobic conditions in order to avoid contamination by bacteria (18). *Picrophilus* spp., which, in contrast to *Thermoplasma* spp., are strictly aerobic, would have been missed with the use of this isolation method.

Phylogenetically, the closest relative of the genus *Picrophilus* is the genus *Thermoplasma*. The organisms in these two genera share several physiological properties; i.e., they grow heterotrophically, preferably on yeast extract as a carbon source, in the same temperature range under strongly acidic conditions (Table 2). Smith et al. (23) have shown that the factor essential for the growth of *T. acidophilum* in yeast extract is most likely a basic oligopeptide, which might also be present in other complex organic carbon sources allowing growth of this organism (18). Like *T. acidophilum*, *Picrophilus* spp. are most likely scavengers utilizing products of decomposition of other organisms.

A source resembling NOB4 both in temperature and in pH, KAW1, yielded *T. acidophilum* rather than *Picrophilus* isolates, indicating that *Picrophilus* and *Thermoplasma* spp. indeed share the same biotope. This is in line with the finding

TABLE 2. Growth conditions and DNA contents of *Picrophilus* and *Thermoplasma* spp.[a]

Species	Temp (°C)			pH			Growth	DNA (mol% G+C)
	Min	Opt	Max	Min	Opt	Max		
P. oshimae	45	60	65	~0	0.7	3.5	Ae	36
T. acidophilum	45	59	63	0.8	2	4	Ae/An	46
T. volcanium	33	59	67	0.8	2	4	Ae/An	38–40

[a] Values for *P. oshimae* are from this study; those for *T. acidophilum* and *T. volcanium* are from reference 18. Abbreviations: Min, minimum; Opt, optimum, Max, maximum; Ae, aerobic; Ae/An, facultatively anaerobic.

that plating of an "authentic" *Thermoplasma* culture under conditions optimal for growth of *Picrophilus* spp. yielded as well isolates of the latter genus, as if the conserved *Thermoplasma* cells derived from a sample which had never been cloned via single colonies had contained *Picrophilus* spp. (Fig. 1).

The lipids of *Thermoplasma* (10) and *Picrophilus* spp. are similar in principle, although those of *Picrophilus* spp. exhibit a simpler pattern. The sugar moiety in the major lipid components of *Thermoplasma* spp., however, is different from the β-glucosyl residue found in the major components of *Picrophilus* spp. In contrast to the lipids of *Sulfolobus* spp., those of *Picrophilus* spp. did not contain nonitol residues.

Like *Thermoplasma* spp., *Picrophilus* spp. appeared to contain cytochromes of the *b* type exclusively (16a).

Besides its hyperacidophily, several characteristics of *Picrophilus* spp. clearly distinguish them from all known strains of *Thermoplasma* and from other archaea. Growth of *Picrophilus* spp. was not observed under anaerobic conditions with or without sulfur, whereas *Thermoplasma* spp. are able to thrive anaerobically, preferably by sulfur respiration (18). In contrast to the archaeal mycoplasmas *Thermoplasma* spp. and like many other archaea, especially thermophiles, both species of *Picrophilus* possess a surface layer. Its structure, however, is different from that of other archaea. It shows tetragonal symmetry as do the layers of *Desulfurococcus* and *Staphylothermus* spp. (16, 27), but it is clearly distinct from these in the details of its architecture. The RNAP of *Picrophilus* spp. showed a subunit pattern different from that of *Thermoplasma* spp., and polyclonal antibodies directed against the *Thermoplasma* RNAP did not cross-react with the *Picrophilus* RNAP. Such cross-reaction usually occurs between members of the same family but not between representatives of different families (our unpublished observations). The extent of the sequence divergence of the 16S rRNA genes, the presence of a distinct S-layer in *Picrophilus* spp., and the lack of cross-reaction of the RNAPs in the immunodiffusion assay are arguments that the genera *Thermoplasma* and *Picrophilus* belong to different families, *Thermoplasmaceae* and *Picrophilaceae*, respectively, within the order *Thermoplasmales*.

The phylogenetic tree of 16S rRNAs shown in Fig. 11 was obtained by considering only transversions within a fraction of 994 conserved positions of the sequences. Although the statistical support (bootstrap values) was not very strong, most of the branching points are supported as well by the results of the parsimony method as by those of the maximum-likelihood method, inferred with fast DNAML (14), and the neighbor-joining (distance) method (7a). The tree shows the genus *Picrophilus* in a neighbor family to the genus *Thermoplasma* in the order *Thermoplasmales*, which forms the next lowest branch above the order *Thermococcales* in the kingdom *Euryarchaeota*. This is in agreement with the position of the genus *Thermoplasma* in phylogenetic trees of DNA-dependent RNAPs and with the fact that like *Thermococcus* spp., *Thermoplasma* and *Picrophilus* spp. do not show the RNAP B component split characteristic for the high branches of the *Euryarchaeota*, including *Archaeoglobus* spp., the methanogens, and the extreme halophiles.

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