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ORIGINAL PAPER

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Thermobaculum terrenum gen. nov., sp. nov.: a non-phototrophic gram-positive thermophile representing an environmental clone group related to the Chloroflexi (green non-sulfur bacteria) and Thermomicrobia

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Abstract A novel bacterium was cultivated from an extreme thermal soil in Yellowstone National Park, Wyoming, USA, that at the time of sampling had a pH of 3.9 and a temperature range of 65–92 °C. This organism was found to be an obligate aerobic, non-spore-forming rod, and formed pink-colored colonies. Phylogenetic analysis of the 16S rRNA gene sequence placed this organism in a clade composed entirely of environmental clones most closely related to the phyla Chloroflexi and Thermomicrobia. This bacterium stained gram-positive, contained a novel fatty-acid profile, had cell wall muramic acid content similar to that of *Bacillus subtilis* (significantly greater than *Escherichia coli*), and failed to display a lipopolysaccharide profile in SDS-polyacrylamide gels that would be indicative of a gram-negative cell wall structure. Ultrastructure examinations with transmission electron microscopy showed a thick cell wall (approximately 34 nm wide) external to a cytoplasmic membrane. The organism was not motile under the culture conditions used, and electron microscopic examination showed no evidence of flagella. Genomic G+C content was 56.4 mol%, and growth was optimal at 67 °C and at a pH of 7.0. This organism was able to grow heterotrophically on various carbon compounds, would use only oxygen as an electron acceptor, and its growth was not affected by light. A new species of a novel genus is proposed, with YNP1^T (T=type strain) being *Thermobaculum terrenum* gen. nov., sp. nov. (16S rDNA gene GenBank accession AF391972). This bacterium has been deposited in the American Type Culture Collection (ATCC BAA-798) and the University of Oregon Culture Collection of Microorganisms from Extreme Environments (CCMEE 7001).

Keywords Thermophile · Green non-sulfur · Chloroflexi · Gram-positive · Yellowstone National Park · Thermal soil

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Introduction

The high-temperature environments in Yellowstone National Park (YNP) are among some of the best studied geothermal features in the world; however, there are relatively few reports concerning microorganisms inhabiting thermal soils in this geothermal complex. Fliermans and Brock (1972) described their observations on the ecology of the sulfur-oxidizing bacteria *Thiobacillus* and *Sulfolobus* in a YNP solfatara region, and, more recently, Norris et al. (2002) employed molecular approaches to elucidate the changes in a YNP soil microbial community resulting from a neo-geothermal heating event.

As part of our efforts to better understand the distribution of thermophilic microorganisms in YNP, we have initiated cultivation and isolation efforts, with one research thrust involving high-temperature soils. This report summarizes cultivation efforts that resulted in the isolation and characterization of a novel extreme thermophile that, based on phylogenetic analysis of the 16S rRNA gene sequence, is a member of a clade that until now has consisted entirely of environmental PCR clones. The most closely related, cultivated and characterized relatives reside in the phyla Chloroflexi and Thermomicrobia.

The Chloroflexi comprises two orders: Chloroflexales, made up of four genera of filamentous gliding phototrophic bacteria, and Herpetosiphonales, which contains a single recognized genus, *Herpetosiphon* (Castenholz 2001). The

phylum Thermomicrobia also contains a single recognized genus, *Thermomicrobium roseum*, which is an obligate chemoorganotroph that forms rose-pink-colored colonies (Jackson et al. 1973). The Chloroflexi contains both thermophiles and mesophiles (Garrity and Holt 2001), whereas *T. roseum* is an extreme thermophile also isolated from YNP (Jackson et al. 1973). The features of the new thermophilic isolate are sufficiently different from those of characterized organisms in both of these phyla that it is described here as a new genus of *Bacteria*, *Thermobaculum*, with the type species *T. terrenum*, gen. and sp. nov.

Materials and methods

Collection site and sampling procedure

Isolate YNP1^T (T=type strain) was isolated from a small depression site, (approximately 2 m in diameter, ~0.5 m deep; located near the confluence of Rabbit Creek and Firehole River) that is heated by an underground source of steam (documented to be thermal for at least 7 years, unpublished data). Soil temperatures at the surface were 65 °C, increasing to 92 °C at the 12-cm depth. Specific chemical features of the soil were determined using analytical methods previously described (Page et. al. 1982; Sparks et al. 1996). Soil samples (~1-5 g) were taken using sterile spatulas, transferred to sterile plastic tubes, and transported back to the laboratory in heated water contained in an insulated bottle.

Isolation, media, and cultivation conditions for YNP1^T

Soil samples were suspended in 0.1 M NH_4PO_4 buffer (65 °C), shaken for 30 min, and then diluted in ten-fold steps. Aliquots of 0.1 ml from each dilution were then spread onto 0.1% yeast extract agar. YNP1^T (T=type strain) was initially identified as visually distinct pink colonies arising after 4 days of incubation at 65 °C. Later tests determined that YNP1^T grew much better on R2A media (Difco, contains 0.5 g $\cdot l^{-1}$ each of starch, glucose, yeast extract, proteose peptone, casamino acids, and 0.3 g l^{-1} sodium pyruvate), and thus R2A was used for subsequent maintenance culturing and for some growth tests. For long-term storage at –75 °C, the organism was grown to mid-exponential phase in R2A broth, diluted with an equal volume of a solution containing 50% R2A broth and 50% glycerol, and then frozen.

For testing anaerobic respiration, the R2A medium was modified to include 5 mM of either nitrate (KNO_3), sulfate (K_2SO_4), ferric iron ($FeCl_3$), or arsenate ($NaAsO_4$) as potential electron acceptors. Anaerobic cultures were inoculated into crimp-sealed serum bottles that were then purged with N_2. Resazurin was included (0.0001%, w/v) as an oxygen indicator.

To examine the potential of YNP1^T to grow chemolithotrophically, the basal medium contained (per 1 distilled water): 1 g NH_4Cl, 0.64 g KH_2PO_4, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g NaCl, 0.1 g yeast extract, 0.006 g $FeCl_3 \cdot 6H_2O$, and 1 ml of trace element solution SL-6 [Medium 27, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)]. The medium pH was adjusted to 7.0. YNP1^T was inoculated into crimp-sealed 20-ml serum bottles with the headspace totaling 50% of total bottle volume. The headspace gas was 90% (by volume) air and 10% CO_2. In some experiments, 50% of the air was replaced by H_2. Elemental sulfur and sulfide were also tested as potential electron donors. Prior to use, the elemental sulfur was heat treated at 100 °C for 30 min. For sulfide, the methods of Nelson (1989) were used to establish anoxic cultivation conditions with opposing vertical gradients of oxygen and aqueous sulfide.

To assess anoxygenic photosynthesis in YNP1^T, serum bottles containing 10 ml basal salts medium (described above for chemolithotrophic growth tests) were incubated anoxically under a tungsten light bulb at 65 °C. The headspace was enriched to 25% CO_2, and either sulfide (0.075% as $Na_2S \cdot 9H_2O$) or H_2 gas (50% headspace) were included. Conditions required for non-sulfur phototrophic bacteria (e.g. purple non-sulfur or *Chloroflexus*) were also tested by providing acetate (0.5% w/v), succinate (0.1% w/v), or ethanol (0.5% v/v) with a trace amount of sulfide (0.005%). In other experiments, these carbon sources were replaced with 2 g l^{-1} yeast extract. All experiments used incandescent lamps.

The optimum pH for growth of strain YNP1T was determined by adjusting the initial medium pH with HCl or NaOH. At the conclusion of each experiment, the initial pH was verified to not have changed during the incubation. Similarly, NaCl tolerance was examined by varying the concentration of NaCl in the medium. The cardinal temperatures for growth were determined in an oil-bath shaker incubator set at the predetermined temperature. Cell doubling times were measured by tracking increases in culture optical density (595 nm). For all growth experiments, cultures were initiated by inoculating with a standardized number of cells (OD_{595}= 0.05), and were done in triplicate. Since the described isolate failed to exhibit growth in defined minimal media (see below), carbon-source utilization was determined by measuring the culture optical density after incubation of a standardized cell inoculum in a dilute yeast extract solution (amended with various carbon compounds) that preliminary experiments had shown would allow for a minimal detectable increase in culture optical density (see below). Catalase, oxidase, nitrate reduction, and fermentation tests employed techniques previously described by Smibert and Krieg (1994).

In the various experiments, YNP1^T was compared to *Escherichia coli* strain K12 (obtained from Dr. Michael L. Kahn, Washington State University), *Pseudomonas aeruginosa* strain PAO1 (obtained from Dr. Daniel Hassett, University of Cincinnati), and *Rhizobium tropici* strain CIAT899 (obtained from Dr. Peter Graham, University of Minnesota). *Bacillus subtilus* strain MSU14 and *Rhodospirillum rubrum* strain MSU358 were obtained from Dr. B. Hudson (Montana State University). *E. coli* was routinely cultivated on LB agar and and *B. subtilus* was grown on nutrient agar (Sambrook et al. 1989), *Rhizobium tropici* was grown on a minimal mannitol medium (Sommerville and Kahn 1983), and *R. rubrum* was maintained phototrophically as described in cultivation instructions supplied by the DSMZ.

Fatty-acid methyl esterase analysis and pigment composition

Fatty-acid methyl esterase (FAME) was measured in 48-h cultures grown in trypticase soy broth at 65 °C. Fatty acids were extracted, methylated with methanolic HCl, and the FAMEs analyzed by gas chromatography. FAME analysis was done by Microbial ID (Newark, Del., USA). Pigment composition was examined with dimethylsulfoxide (DMSO)-extracted cells. Late-exponential-phase cultures were pelleted by centrifugation, washed with 0.85% saline solution, and resuspended in DMSO. After extracting at room temperature for 30 min., the suspension was centrifuged for 3 min at 13000×*g* and the supernatant was used to record the absorption spectrum in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Palo Alto, Calif., USA).

Cell wall characterization

To characterize the cell wall of YNP1^T, the muramic acid content was measured and compared to that of *E. coli* and *B. subtillis*. In addition, lipopolysaccharide (LPS) content was examined as a marker for the presence of an outer membrane. Peptidoglycan was extracted using the methods of De Jonge et al. (1992). To quantify the amount of muramic acid in the cell walls, the peptidoglycan preparations were hydrolyzed in 3 N HCL for 4 h at 95 °C, neutralized with 3 N NaOH, and the muramic acid content determined by the colorimetric method of Hadzija (1974). Lipopolysaccharide (LPS) was extracted as described by Sprott et al. (1994). Briefly, cells were grown to late exponential phase, pelleted, and then resuspended in 50 ul SDS-PAGE lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 1 M

Tris-HCL buffer, pH 6.8) and incubated at 100 °C for 10 min. Proteinase K (25 µg) was then added and the samples were incubated at 60 °C for 1 h. To purify the LPS, proteins were extracted with an equal volume of 90% phenol at 65 °C for 15 min; the aqueous phase was extracted with ten volumes of ethyl ether and then mixed with an equal volume of SDS-PAGE lysis buffer. LPS components were then resolved in Tricine-SDS (18%) polyacrylamide gels and visualized by silver staining (Sprott et al. 1994).

DNA characterization, 16S rDNA sequencing, and phylogenetic analysis

DNA G+C content was determined from purified nucleic acids using an extraction methodology adapted from Mazel et al. (1986). Acid-washed glass beads (0.5 g, 106 µm diameter; Sigma, St. Louis, Mo., USA), 33.3 µl 20% SDS, 167 µl 3% diatomaceous earth (Sigma), and 583 µl Tris-buffered phenol were added to a frozen cell pellet. The sample was shaken for 160 s on a bead mill and centrifuged in the cold for 15 min at 14,000×*g*. The aqueous layer was transferred to a fresh tube, and nucleic acids were precipitated at –20 °C with 3 M sodium acetate (pH 5.2) and 95% ethanol. After centrifugation (14,000×*g*), the resulting nucleic acid pellet was washed with 70% ethanol and suspended in 100 µl nuclease-free water. Nucleic acids were RNAse treated and then purified using standard phenol-chloroform purification. G+C content was quantified using HPLC as described by Mesbah et al. (1989) using non-methylated λ DNA as a standard. For sequencing the 16S rRNA gene, the near full-length 16S rRNA gene was amplified using purified DNA as template, along with the *Bacteria*-specific primer 8F and the universal primer 1492R (Amann et al. 1995). The purified PCR product was sequenced using the ABI Prism BigDye Terminator Cycle Sequencing reaction kit and an ABI 310 DNA sequencer (Applied Biosystems, Foshi City, Calif., USA). Primers used for sequencing included the 8F and 1492R primers, and internal primers which included 1070F (Ferris et al. 1996), 338F, 338R, 522F, 522R, and 785F (Amann et al. 1995; Jackson et al. 2001). For phylogenetic analysis, an initial BLAST (Altschul et al. 1990) search of public databases approximated the phylogenetic affiliation of YNP1^T and identified closely related sequences, which were downloaded and aligned using ClustalW (Thompson et al. 1994). Phylogenetic trees were constructed using the parsimony treeing program in the PAUP (http://paup.csit.fsu.edu) software package.

Light and electron microscopy

Direct examination by light microscopy was carried out using a Zeiss Axioskop 2 microscope, with images acquired using a Zeiss Axiocam digital camera. Electron microscopy was done using a Zeiss 100 CA transmission electron microscope operating at 100 kV. Thin sections of cells were prepared by mixing a pellet of exponential-phase cultures with 2% Noble agar. This allowed the cells to be easily handled during the rest of the fixation and embedding procedure. Cells were fixed using 3% glutaraldehyde in potassium sodium phosphate buffer (PSPB), pH 7.2, and washed three times with PSPB before further fixation and staining in 2% osmium tetroxide for 4 h at room temperature. Dehydration was then completed with a graded ethanol series followed by addition of the transitional solvent propylene oxide. The cells were then infiltrated, embedded in Spurr's resin (Spurr 1969), and stained with uranyl acetate/Reynolds' lead citrate (Reynolds 1963).

Results

Morphological characteristics

Strain YNP1^T was isolated from a soil that had a pH of 3.9 (measured in a suspension containing one part soil to two parts distilled water), 109 g soluble organic carbon kg soil^{-1}, 151 mg NH_4^+ · kg soil^{-1}, and temperatures ranging from 65 to 92 °C. The strain was first identified as a 2–3-mm diameter, pink-colored colony arising on 0.1% yeast extract agar, and was repeatedly subcultured to obtain a pure clonal isolate. It is a non-motile rod (2-3 µm in length, 1–1.5 µm in diameter) that stains gram-positive in exponential-phase cells. Cells occur singly or in pairs and were not light refractory (Fig. 1), indicating that this organism does not form endospores under the growth conditions employed in this study. Electron microscopy of exponentially growing and stationary-phase cells also failed to identify spore-like structures, but did reveal a cell wall structure that appeared typical for gram-positive bacteria (Fig. 2A). A single cytoplasmic membrane was located internal to a cell wall that averaged approximately 34 nm in

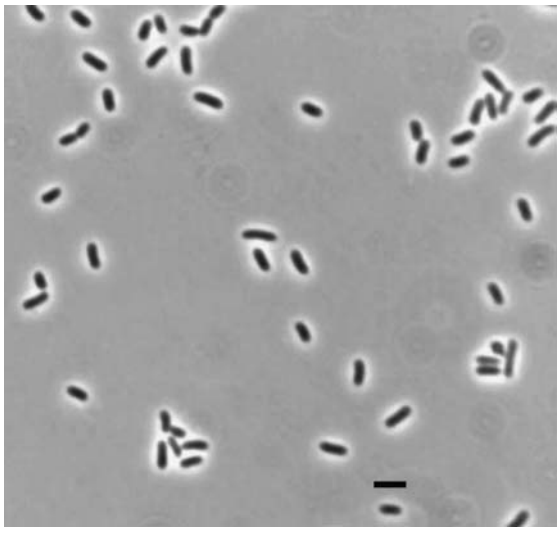

Fig. 1 Phase-contrast image of strain YNP1^T. *Scale bar* 2 µm

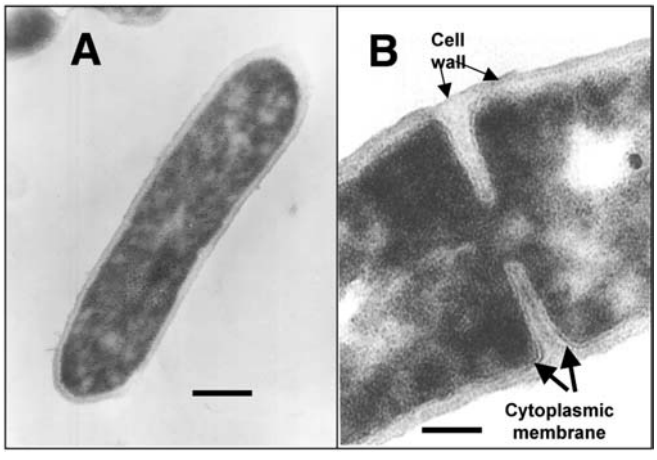

Fig. 2A, B Transmission electron micrographs of *Thermobaculum terrenum*. **A** Lower magnification (56,000×) thin section contrasted with lead citrate and uranyl acetate. *Bar* 0.25 µm. **B** Higher magnification (195,000×) thin section of the cell wall ultrastructure of a dividing cell, also contrasted with lead citrate and uranyl acetate. *Bar* 0.1 µm

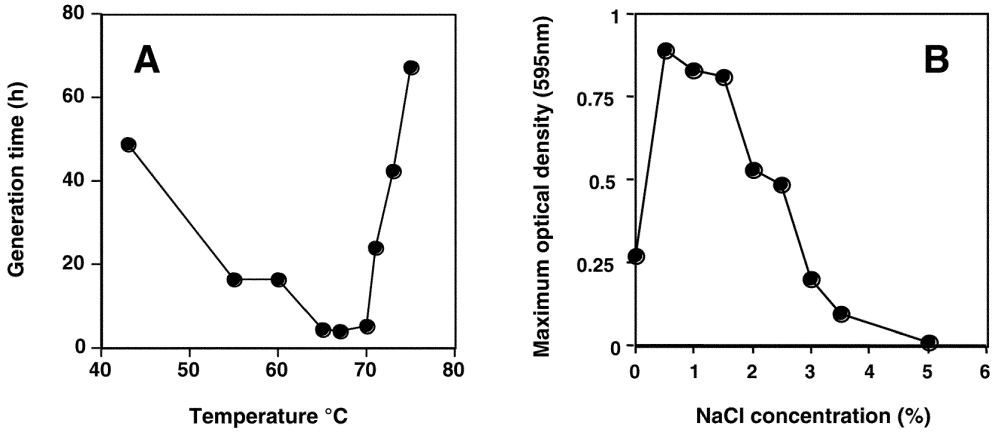

Fig. 3A; B Influence of incubation temperature, and medium NaCl concentration on growth of strain YNP1^T. **A** Culture doubling time as affected by incubation temperature. Cultures were incubated for up to 8 days. **B** Culture growth as affected by NaCl concentration. Cultures were grown for 120 h and culture optical density measured (595 nm). Error bars represent one standard error of the mean of at least three replicate cultures but were smaller than symbols and are thus not shown

thickness (Fig. 2B), and no outer membrane structure was visible (Fig. 2B).

Growth experiments

Optimum growth occurred in a temperature range of 65–70 °C (Fig. 3A), with T_{opt}=67 °C, T_{min}=43 °C, and T_{max}=75 °C. Culture doubling time at T_{opt} was 4.05 h and increased sharply above 70 °C. Growth at the temperature extremes was relatively poor; the culture doubling times for T_{min} and T_{max} were 49 h and 67.6 h, respectively (Fig. 3A). Growth occurred in a pH range of 6–8, the optimum being pH 7, and maximum growth occurred when the R2A medium was amended with 0.5% NaCl (Fig. 3B). Strain YNP1^T was positive for catalase, urease, and nitrate reduction, but tested negative for oxidases, and was also negative for fermentation of glucose or lactose. Parallel tests showed that the positive and negative control organisms *E. coli*, *P. aeruginosa*, and *R. tropici* behaved according to previously published reports (results not shown).

To attempt to account for any potential oligotrophy-like growth preferences, strain YNP1^T was incubated in various dilutions (0.25×, 0.5×, 1.0×) of the defined minimal salts medium used in this study. No growth was observed in defined medium; however, YNP1^T readily grew on complex media such as yeast extract agar or R2A agar. Therefore, for the purpose of identifying carbon sources that support growth, the minimal concentration of yeast extract required to support minimal detectable growth in broth was determined (0.01% w/v) and was assumed to be required for supplying a needed growth factor (e.g. amino acid and or vitamin). Under oxic culture conditions, glucose, fructose, ribose, sorbitol, sucrose, xylose, or xylitol supported significant culture growth beyond that attributable to the dilute yeast extract alone (Table 1). Other tested carbon sources, such as polyols, major amino acids, and organic acids, failed to stimulate growth (Table 1). No anaerobic growth in R2A broth (contains various possible carbons sources used aerobically) was observed in the presence of sulfate, nitrate, ferric iron, or arsenate as possible electron acceptors. Furthermore, no chemolithoautotrophic growth was observed in an experimental matrix that included the electron donors H_2, H_2S, or S^0 with oxygen as electron acceptor. Light (including irradiance from tungsten lamps) did not enhance growth under any of the conditions (oxic or anoxic) tested.

Table 1 Survey of carbon utilization by isolate YNP1^T. Carbon compounds tested are grouped according to those that readily supported growth beyond background levels observed with 0.01% yeast extract, and those that either did not support growth or that did so only poorly. Culture optical density values are representative results from one of two experiments, in which a single flask culture containing one of the listed carbon sources was inoculated to a starting optical density of 0.015–0.20 and then incubated for 3 days. All carbon sources were added at 5 g l^{-1}

Compound	Culture optical density
Distilled water	0.003[a]
0.01% yeast extract	0.018
Utilized	
Fructose	0.099
Glucose	0.151
Ribose	0.170
Sorbitol	0.075
Sucrose	0.154
Xylose	0.243
Xylitol	0.152
Poorly or not Utilized	
Acetate	0.023
Alanine	0.017
Arabinose	0.028
Citrate	0.016
Glutamate	0.012
Malate	0.014
Mannitol	0.031
Succinate	0.025

[a]No growth occurred, optical density represents values resulting from inoculum

Fatty acids analysis

Sherlock computer software (Microbial ID) was used to characterize the FAME profile of YNP1^T. Major fatty

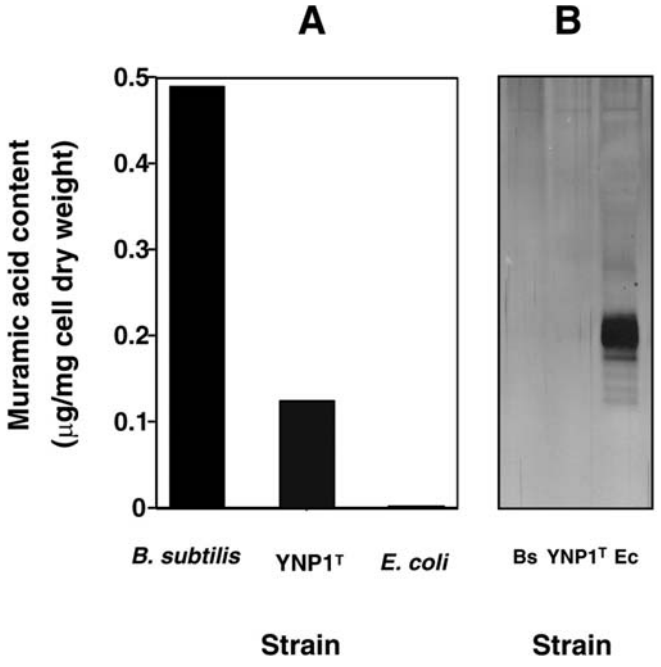

Fig. 4 Comparison of cell wall and outer membrane features of YNP1^T with *Escherichia coli* and *Bacillus subtillis*. **A** Cell muramic acid content, **B** silver stain detection of lipopolysaccharide (LPS) extracts of strain YNP1^T, *B. subtillis*, and *E. coli* separated in a polyacrylamide gel. Results for **A** and **B** are from one of two independent experiments that demonstrated the reproducibility of these observations

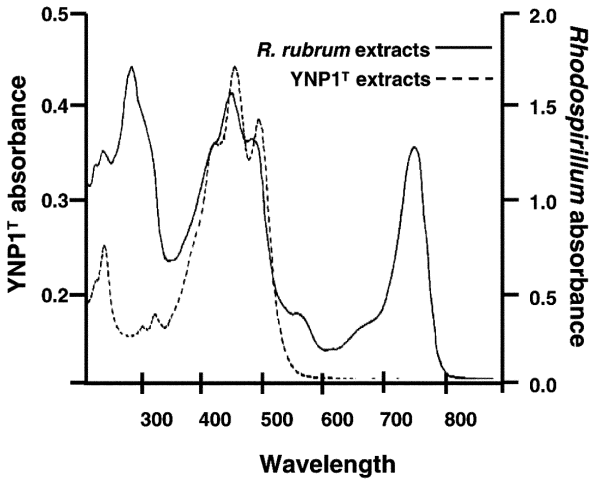

Fig. 5 Absorption spectra of dimethylsulfoxide extracts of strain YNP1^T and *Rhodospirillum rubrum* strain MSU358 cultures. Cells of strain YNP1^T were grown aerobically in R2A medium, whereas *Rh. rubrum* was cultivated photoheterotrophically in medium 27 (DSMZ) at 25 °C. Spectra are reproducible and are representative scans for both organisms

acids included 18:0 (27% of total); 17:0 iso (11.6%); 19:0 iso (12.9%); 18:0 anteiso (12.5%); 20:0 (16.5%); and 19:0 (6.6%). Other fatty acids (16:0, 16:0 iso, 17:0 anteiso, 18:0 iso, and 20:0 iso) ranged from 0.25 to 5.25% of total fatty acids.

Cell wall characterization

Transmission electron microscopy showed evidence of a thick peptidoglycan layer (Fig. 2B) consistent with the monoderm cell wall structure (Gupta 1998) of a classic gram-positive bacterium.Additional information regarding the cell wall structure of strain YNP1^T was obtained by biochemical analysis. The muramic acid content of strain YNP1^T was roughly 25% of that measured for *B. subtillus* (Fig. 4A) but almost 40-fold greater than in *E. coli*, providing biochemical evidence that was consistent with strain YNP1^T having a gram-positive-like cell wall structure. Furthermore, LPS was not detected in strain YNP1^T upon silver staining of materials derived from typical LPS extraction protocols and separated in denaturing polyacrylamide gels (Fig. 4B). The same LPS extraction and separation experiments with the positive control organism *E. coli* yielded silver stain patterns consistent with oligosaccharide side-chain and core components similar to that previously known for *E. coli* and other gram-negative bacteria (Summers et al. 2000).

Pigments

Organic solvents, such as chloroform, ethanol, methanol, and acetone, failed to extract the pink pigment associated with strain YNP1^T. However, the pink pigment readily extracted with DMSO and remained soluble. The pigment exhibited significant absorption at wavelengths 267, 326, 399, 483, 511, and 549 nm (the latter three peaks suggesting a carotenoid similar in absorption properties to spirilloxanthin), but not at wavelengths that would be typical for phototrophs, such as a bacteriochlorophyll a (Fig. 5) or bacteriochlorophylls a and c as in *Chloroflexus* (Pierson and Castenholz 1974b). As a comparison, the purple nonsulfur bacterium, *R. rubrum* (which contains spirilloxanthin) was similarly extracted and found to release DMSO-soluble pigments that absorbed at 367, 608, and 775 nm, which are consistent with bacteriochlorophyll *a* absorbance, and at 479, 509, and 544 nm (Fig. 5), which are properties of the carotenoid spirilloxanthin (Pfennig and Trüper 1992).

DNA characterization, 16S rDNA sequencing and phylogenetic analysis

HPLC analysis of purified and hydrolyzed DNA from strain YNP1^T determined the G+C content to be 56.4%. Phylogenetic analysis of the PCR-amplified 16S rRNA gene (GenBank accession AF391972) included 1,150 nucleotides, truncated to that of the environmental clone H1.43.f (accession number AF005749), which was the closest database match to strain YNP1^T. Parsimony analysis placed strain YNP1^T within a clade comprised of PCR-amplified environmental clones that are most closely related to the phyla Chloroflexi and Thermomicrobia (Fig. 6). Examina-

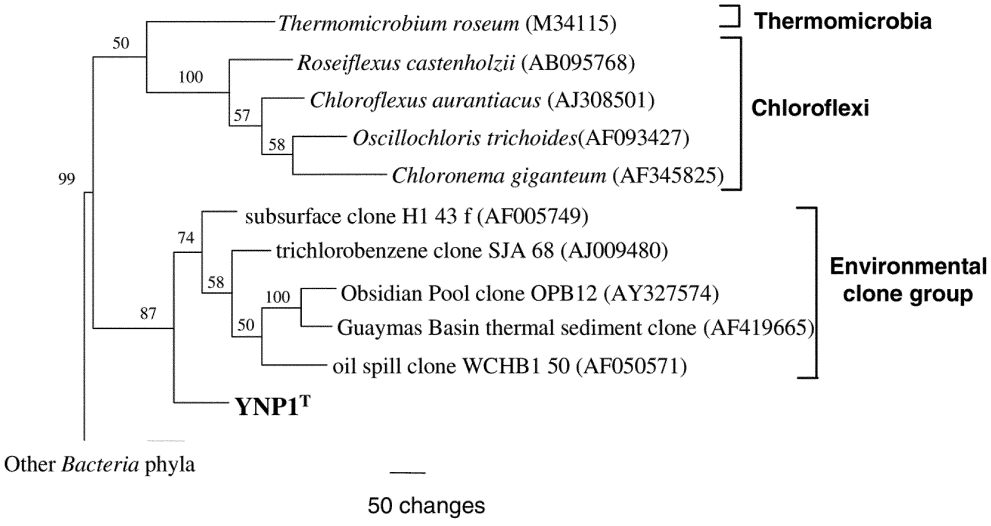

Fig. 6 A parsinomy-based dendrogram showing phylogenetic relationships of 16S rRNA genes from strain YNP1^T, cultured and characterized organisms in the phyla Chloroflexi and Thermomicrobia, and related PCR clones obtained from various environments. Representatives of other *Bacteria* phyla used as an outgroup included *Thermotoga hypogea* (U89768), *E. coli* (AP002564), *Mycobacterium pinnipedii* (AF502574), *B. subtilis* (Z99119.2), *Azospirillum brasilense* (X79739). Bootstrap values, expressed as a percentage of 500 replications, are given at branch points. *Bar* Branch lengths equivalent to 50 changes

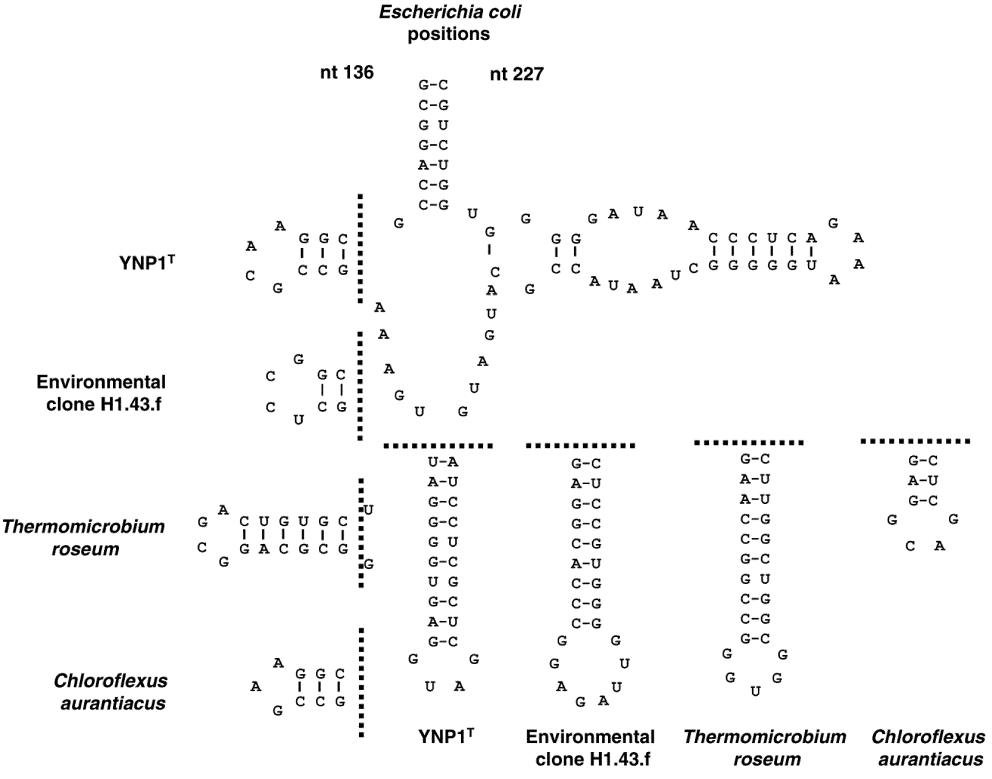

Fig. 7 Analysis of putative stem-loop structures in the nucleotide 136–227 region (*E. coli* numbering) of the strain YNP1^T 16S rRNA molecule, and comparison to phylogenetically closely related bacteria (cultured and characterized organisms or environmental clones)

tion of potential secondary structure differences showed that strain YNP1^T contains two stem-loop structural features, between nucleotides 136 and 227 (*E. coli* numbering), in the region of the 16S RNA molecule that were more similar to those modeled for environmental clone H1.43.f than for either *T. roseum* or *Chloroflexus aurantiacus* (Fig. 7), which are characterized representatives of the phyla Thermomicrobia and Chloroflexi, respectively.

Discussion

Phylogenetic analysis of the 16S rRNA gene of strain YNP1^T placed this organism into a clade consisting of environmental clones, and separate from the closest related, cultivated and characterized organisms belonging to the phyla Chloroflexi and Thermomicrobia (Fig. 6). Comparisons of putative stem-loop secondary structures supported the phylogram placement, suggesting that strain YNP1^T is most closely related (although still distant) to an organism represented by the environmental PCR clone H1.43.f (86.4% similarity). Clone H1.43.f was amplified from DNA extracted from a deep subsurface non-thermal environment (Chandler et al. 1998), and other environmental clones in this clade were also retrieved from non-thermal environments (river sediments and an aquifer), in some cases contaminated with hydrocarbons and or chlorinated solvents (Dojka et al. 1998; von Wintzingerode et al. 1999).

Table 2 Comparison of novel isolate YNP1^T with *Thermomicrobium roseum*. Information for *T. roseum* is from Jackson et al. (1973)

Feature	YNP1^T	*T. roseum*
$Temp_{opt}$	67 °C	70–75° C
pH_{opt}	7.0	8.2–8.5
mol% G+C	54.6	64.3
Morphology	Straight rod	Pleomorphic
Cell envelope	Monoderm[a]	Diderm[a]

[a]Monoderm refers to single membrane; diderm refers to double membrane (Gupta 1998)

Another clone in this group, OPB12, was amplified from Obsidian Pool, a circumneutral pH, high-temperature environment, in Yellowstone (Hugenholtz et al. 1998) and shared a node with a clone amplified from DNA extracted from Guaymas Basin thermal sediments (Teske et al. 2002). The occurrence of mesophilic and thermophilic organisms in this environmental clone group is similar to that documented for the Chloroflexi (Pierson 2001). The phylogenetic placement of strain YNP1^T separate from *T. roseum* is consistent with other major differences between these organisms (Jackson et al. 1973), which are summarized in Table 2. Temperature and pH optima differ significantly, and more importantly, chemotaxonomic characteristics such as DNA mol% G+C content, morphology, and cell envelope characteristics, all differ markedly between these two bacteria.

Isolate YNP1^T was capable of heterotrophic growth on several carbon sources when the medium was amended with a low concentration of yeast extract (Table 1). The need for "growth factor" concentrations of yeast extract is similar to that observed for previously described Chloroflexi organisms such as *Chloroflexus aurantiacus* (Pierson and Castenholz, 1974b). However, no evidence of photoheterotrophy was observed in strain YNP1^T, even with high concentrations of yeast extract (2 g l^{-1}), which supports abundant growth of *Chloroflexus* (Pierson and Castenholz 1974a). In all cases, strain YNP1^T displayed an absolute requirement for oxygen, with no growth observed in the presence of a variety of potential alternative electron acceptors supplied at levels sufficient to support anaerobic respiration and growth.

The majority of GenBank accessions describing 16S rRNA genes that branch into a general assemblage comprising the Chloroflexi and Thermomicrobia phyla (previously collectively referred to as the green non-sulfur bacteria) are represented by PCR-amplified environmental clones (results not shown). The relatively few isolated and characterized bacteria in this large group display a range of metabolic lifestyles (Pierson and Castenholz 2001), including both phototrophy (Pierson and Castenholz 2001) and chemoheterotrophy (Holt and Castenholz 2001; Jackson et al. 1973). Therefore, the apparent obligate heterotrophic nature of strain YNP1^T is not inconsistent with other organisms in this group, described by Oyaizu et al. (1987) as an "incongruous assemblage". A heterotrophic metabolism would probably be suitable for this organism in the soil environment from which it was isolated, as soluble carbon was relatively abundant. The optimum growth temperature of strain YNP1^T (Fig. 3A) is also consistent with the sampled soil (65–92 °C), although the pH optimum of strain YNP1^T was somewhat surprising given the clearly acidic reaction of this soil. In pure culture, strain YNP1^T failed to grow at pH 4.0 or even 5.0, suggesting that this particular thermal soil habitat is not optimal for strain YNP1^T. Also, strain YNP1^T does not apparently form spores that could otherwise help explain survival under these conditions. We note, however, that even though populations of strain YNP1^T were very low (agar plate counts ~100 colonies g $soil^{-1}$), we were able to re-isolate it from the same soil (unpublished data), suggesting that there are at least small populations of strain YNP1^T that were somehow introduced into this soil and remained viable. Regardless, strain YNP1^T is likely not very competitive under these conditions. The relatively low salt-tolerance of strain YNP1^T suggests that this particular species would not be well adapted to marine environments.

The pink pigment of strain YNP1^T was the visually distinguishing feature that allowed it to be readily recognized on the original isolation agar plates. The pigment was only extractable with DMSO and did not absorb at wavelengths associated with bacteriochlorophylls or the carotenoids of *Thermomicrobium* or of the Chloroflexi. However, the pigment did absorb at wavelengths typical of the carotenoid pigments of the spirilloxanthin series or tetradehydrolycopene (Britton 1985), and in this regard closely matched the absorption pattern of *R. rubrum*, used as a positive control organism in these particular experiments. While a definitive identification of the pink pigment as spirilloxanthin has not been made, the *R. rubrum* DMSO extracts in this study matched previously characterized spectra for this organism in other solvents and was also consistent with the presence of spirilloxanthin previously shown to occur in *R. rubrum* (Pfennig and Trüper 1992). An interesting aspect of this possible carotenoid match is that *R. rubrum* is a member of the α-Proteobacteria, which is only distantly related to the proposed phylogentic position for strain YNP1^T.

Perhaps the most intriguing characteristic of strain YNP1^T that sets it apart from Chloroflexi and its relatives is the cell wall structure, which appears to resemble that of a typical gram-positive bacterium. The Chloroflexi all share the common trait of lacking an outer membrane (Castenholz 2001); however, all members also have a very thin cell wall and stain gram-negative (Pierson and Castenholz 2001; Holt and Castenholz 2001), whereas *T. roseum* has a typical gram-negative cell wall architecture and also stains gram-negative (Perry 2001). Therefore, strain YNP1^T likely represents an extreme example within a continuum of cell wall structures observed in the Chloroflexi-Thermomicrobia cluster.

Description of *Thermobaculum* gen. nov.

Thermobaculum, Ther.mo.ba'cu.lum Gr. adj. thermos hot; L. neut. n. baculum small rod, N.L. neut. n.; *Thermobaculum*: hot small rod. Rod-shaped and occurring singly or in pairs, isolated from a geothermally heated soil. Cells stain gram-positive. Growth is strictly aerobic and heterotrophic. Based on analysis of 16S rDNA sequence, *Thermobaculum* is phylogenetically most closely related to organisms currently represented by environmental PCR clones, with the closest characterized isolates belonging to the phyla Chloroflexi and Thermomicrobia. Type species: *Thermobaculum terrenum*.

Description of *Thermobaculum terrenum* sp. nov.

T. terrenum ter.re' num L. neut adj. terrenum belonging to earth/soil. Cells are non-motile, measuring 1–1.5×2–3 μm, and are enveloped by a thick cell wall (~34 nm with transmission electron microscopy) external to a cytoplasmic membrane. Colonies are pink in color. Growth occurs between 41 and 75 °C (optimum 67 °C), at pH 6–8 (optimum 7.0), and optimally in complex media containing 0.5% NaCl. Growth on yeast extract [required for growth factor(s)], fructose, glucose, ribose, sorbitol, sucrose, xylose, and xylitol. Membrane composed primarily of straight-chain and branched fatty acids, murein present in large amounts consistent with thick cell wall, 56.4 mol% G+C. The type strain YNP1^T has been deposited in the American Type Culture Collection as accession number ATCC BAA-798 and in the University of Oregon Culture Collection of Microorganisms from Extreme Environments as accession number CCMEE 7001.

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