Bacillus infernus sp. nov., an Fe(III)- and Mn(IV)-Reducing Anaerobe from the Deep Terrestrial Subsurface

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Bacillus infernus sp. nov. was isolated from ca. 2,700 m below the land surface in the Taylorsville Triassic Basin in Virginia. *B. infernus* was a strict anaerobe that grew on formate or lactate with Fe(III), MnO₂, trimethylamine oxide, or nitrate (reduced to nitrite) as an electron acceptor, and it also grew fermentatively on glucose. Type strain TH-23 and five reference strains were gram-positive rods that were thermophilic (growth occurred at 61°C), halotolerant (good growth occurred in the presence of Na⁺ concentrations up to 0.6 M), and very slightly alkaliphilic (good growth occurred at pH 7.3 to 7.8). A phylogenetic analysis of its 16S rRNA indicated that *B. infernus* should be classified as a new species of the genus *Bacillus*. *B. infernus* is the only strictly anaerobic species in the genus *Bacillus*.

Interest in the microbiology of the terrestrial subsurface has increased rapidly since sizable populations of viable microorganisms were found, first in relatively shallow aquifers (for a review, see reference 16) and later in much deeper environments as well (15). Subsurface microorganisms are often viewed as important because they might affect the fate and transport of toxic contaminants in their environment and also because they could play a significant role in subsurface geochemical processes. Moreover, subsurface microbial communities include strains with novel metabolic properties potentially useful to industries or in bioremediation or biotechnology (13, 14).

Populations of viable microorganisms have been found to exist hundreds of meters below the surface in geologically and hydrologically diverse subsurface environments, including the Atlantic coastal plain aquifers in South Carolina (15), unsaturated and saturated sediments and basalts in arid regions of Idaho and Washington (6, 21), unwelded volcanic tuffs in Nevada (1), and crystalline bedrock in Sweden (27, 28). The microbial communities in these different environments vary in size, diversity, composition, metabolic properties, and other characteristics, indicating that their habitats are controlled by interactions of geochemical, hydrologic, and microbiological factors. These interactions are of concern not only to elucidate the ecology of the deep subsurface but also to better understand processes affecting fossil fuel evolution, to define the environments of proposed subsurface waste repositories, and to predict the fate of subsurface contaminant plumes.

At the present time, little is known about the origins of the microorganisms found in various subsurface environments. It is not clear whether these organisms have survived in situ since the deposition of their host sediments or whether they were transported to the sediments more recently by percolation of meteoric water from the surface. Preliminary research on the origins of microbes in the deep terrestrial subsurface may form

the basis for understanding how subsurface ecosystems develop and function; this research has been conducted since 1992 by the U.S. Department of Energy's Deep Microbiology Subprogram (13). As a part of this research, samples of organic compound-rich, laminated shales and low-porosity cemented sands were obtained from depths of 2.65 to 2.77 km in the Taylorsville Triassic Basin in Virginia. These samples were examined for the presence of viable microbes. Geological evidence suggests that microbes inhabited these depths between 200×10^6 and 140×10^6 years ago, when penetration of meteoric water flow into the basin was probably greatest. Since then, most groundwater flow has been funneled preferentially through the overlying highly permeable sediments. This would make highly unlikely any subsequent introduction of microbes, which would have to be transported through 2.5 km of low-permeability and low-porosity sedimentary rock (3). Consequently, there is a good chance that the viable microorganisms detected in these materials have survived in situ for a long time.

In this paper we describe the characteristics of an unusual bacterium that was isolated from one of the Taylorsville shale samples. This bacterium is a strictly anaerobic *Bacillus* species that can grow by fermenting glucose or by oxidizing formate or lactate while reducing electron acceptors such as MnO₂ and Fe(III).

(Portions of this work have been presented previously [24].)

MATERIALS AND METHODS

Inoculum for isolation of new strains. Subsurface samples were obtained as part of an experiment to determine whether viable bacteria exist in geological strata that probably have been isolated hydrologically from the surface for more than 10^8 years. Side wall core samples were obtained from 2.7 km below the land surface in the Taylorsville Triassic Basin in Virginia. These samples consisted of a fine-grain, laminated siltstone with abundant organic matter and were crosscut by carbonate veins. The samples appeared to be samples of a lake sediment that had not been bioturbated (3). The in situ conditions for these samples were estimated to be thermic (60°C), brackish (1.2% NaCl), and anoxic (3). The formation cooled to its present temperature about 1.4×10^8 years ago and may have been hydrologically isolated since that time (3). The samples were immediately placed in a chamber containing an inert atmosphere, pared to remove the potentially contaminated surface portion, and then shipped on ice to a laboratory within 24 h. The samples were inoculated into enrichment cultures for a variety of bacterial physiological groups. The viable microbes detected in the core

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samples were predominantly anaerobic thermophilic bacteria, including fermentative, iron-reducing, sulfate-reducing, and denitrifying bacteria.

The possibility that these organisms were the result of contamination during the drilling and sampling process was eliminated by several lines of evidence. The most likely source of contamination was the circulating drilling muds used during the drilling process. These muds contained 10^6 CFU of aerobic mesophilic heterotrophs, predominantly *Pseudomonas stutzeri*, per milliliter. Neither *P. stutzeri* nor any other aerobic organism was detected in the core material, indicating that the number of drilling mud contaminants was reduced by a factor of 10^{-6} or smaller. The numbers of microorganisms detected in the cores were too great to be a result of contamination by the muds (35). The total phosphorylated lipids were extracted from the samples, but the composition of these lipids was distinctly different from the compositions of the lipids found in drilling muds, surface sediments, or other likely sources of contamination. Conservative chemical tracers introduced into the drilling fluids were not detected in samples, confirming that the samples were not contaminated (3).

Source of cultures and culture methods. The following microbial strains were obtained from the American Type Culture Collection (ATCC): *Bacillus benzeovorans* B-1^T (= ATCC 49005^T) (T = type strain), *Bacillus circulans* 26^T (= ATCC 4513^T), *Bacillus firmus* 613^T (= ATCC 14575^T), and *Bacillus lentus* 670^T (= ATCC 10840^T). These strains were routinely grown on nutrient agar, and the incubation temperature was always 37°C. The strictly anaerobic strains which we isolated were maintained by using the anaerobic techniques of Hungate (18). These cultures were grown at 55°C in MSA medium, which was identical to MS medium (4) except that the N₂-CO₂ gas mixture (7:3) was replaced by pure N₂ to raise the pH to 8.0. MS medium is an anoxic medium with a bicarbonate and CO₂ buffer system and contains minerals, yeast extract, peptones, and mercaptoethanesulfonate as a reducing agent (4). We added 20 mM formate, 20 mM acetate, and 40 mmol of FeCl₃ per liter to MSA medium to grow the newly isolated strains. The medium used for enrichment was MSA medium with the concentrations of yeast extract and peptone reduced to 0.5 g/liter, with mercaptoethanesulfonate omitted, and with the following additions: 20 mM formate, 20 mM acetate, and 20 mM MnO₂ as catabolic substrates and 10 g of NaCl per liter. The MnO₂ was prepared by mixing a solution containing 95 g of KMnO₄ per liter with an equal volume of a solution containing 178 g of MnCl₂ · 4H₂O per liter (25). Roll tubes were used for isolation (18). The medium used for isolation was identical to the enrichment medium, except that it did not contain NaCl but did contain 18 g of purified agar per liter.

Where indicated below, the pH of the culture medium was adjusted to values greater than 8.0 by adding Na₂CO₃ and the pH was adjusted to values less than 8.0 by exchanging the gas phase with a mixture of N₂ and CO₂.

Measurement of growth. Growth in MSA medium supplemented with formate and FeCl₃ was detected by the production of a gray precipitate, apparently siderite (FeCO₃), which provided a reliable visual indication of ferric reduction. Growth was monitored by measuring Fe(II) production and calculating specific growth rates by using the software package TableCurve 2D, version 2.00 (AISN Software, Inc.), to determine the least-squares fit of the Gompertz equation (17, 38) to the amount of accumulated Fe(II). The optimum temperature was determined with the TableCurve 2D software by a least-squares fit of the square-root model (30) to the growth rates measured at various temperatures.

Physiological tests. Aerobic growth was examined by inoculating and incubating agar shake cultures (nutrient agar supplemented with 1 g of glucose per liter). Molten agar medium (45°C) was inoculated, cooled to solidify the agar, and incubated at 50°C. Growth was monitored daily by visual observation. The use of carbohydrates as catabolic substrates was tested by inoculating cultures into MSA medium containing 1 g of the carbohydrate being tested per liter. Growth was measured by determining A₆₀₀. The ability to use electron donors was tested by inoculating strains into MSA medium containing 20 mM FeCl₃ plus a potential electron donor at a concentration of 10 mM (or 50 kPa of H₂). We monitored the use of electron donors by measuring the formation of Fe²⁺ in these cultures. The ability of strains to use electron acceptors was tested by inoculating strains into MSA medium containing 20 mM formate plus a potential electron acceptor at a concentration of 10 mM. Most growth was monitored by determining A₆₀₀ and was compared with the growth of controls; the use of Fe³⁺ and MnO₂ as electron acceptors was monitored by measuring Fe²⁺ and Mn²⁺ contents.

Other physiological tests were performed by using previously published procedures; for the strictly anaerobic strains isolated in this study, the tests were modified by incubating preparations in a 100% N₂ atmosphere. Cultures were tested to determine whether they hydrolyzed starch, gelatin, and casein (33); the organisms used as controls in these tests were *B. benzeovorans*, *B. circulans*, *B. firmus*, and *B. lentus*. Reduction of nitrate to nitrite was measured in nitrate broth (33) by using the same four organisms as controls. Production of nitrogen gas and ammonia was also measured after growth in nitrate broth (33). Ammonia contents were measured with Nessler's reagent.

Microscopy. Gram staining was performed by the Hucker method (9). Cells used for thin-section electron microscopy were fixed at room temperature for 30 min in 2.5% glutaraldehyde buffered to pH 7.4 with 0.2 M sodium cacodylate. The cells were then postfixed in osmium tetroxide for 30 min at 4°C, dehydrated, and embedded in Spurr low-viscosity resin. Sections were poststained with uranyl acetate and lead citrate and examined.

Oligotrophic survival. Late-logarithmic-phase cells in MSA medium supplemented with 10 mM glucose were collected by centrifugation at 20,000 × g for 20

min. The pellet was washed twice and suspended in anoxic 100 mM bicarbonate buffer (pH 8). This suspension contained about 5×10^7 viable cells per ml. Cell numbers were determined by performing a three-tube, most-probable-number analysis in MSA medium supplemented with 10 mM glucose. Inside an anaerobic chamber, samples of the suspension were placed in sterile 1-ml glass ampoules that had 5-cm-long pieces of latex tubing attached to their openings. After the samples were added, the ampoules were temporarily sealed with pinch clamps on the latex tubing. The ampoules were removed from the anaerobic chamber and individually heat sealed with a small torch. Just before sealing, a syringe needle was inserted through the tubing to allow excess gas to escape during the heating process. After the heat-sealed ampoules cooled, they were incubated in a water bath. Periodically, ampoules were randomly selected for most-probable-number enumeration.

Phylogenetic analysis. DNAs from strains TH-22 and TH-23^T were isolated by the chloroform-isoamyl alcohol procedure (19). Approximately 20 ng of DNA was used as a template for PCR amplification (31) of an approximately 1,500-base segment of the 16S rRNA gene (i.e., nearly the entire gene). The PCR amplification primers (37) used were primer fd1 (AGAGTTTGATCCTGGCT CAG) and primer rp2 (ACGGCTACCTTGTTACGACTT). The PCR amplification products were sequenced with an Applied Biosystems model 373A DNA sequencer by using the Taq DyeDeoxy terminator cycle sequencing method (2, 26). The following primers were used for sequencing: primer C (ACGGGCG GTGTGTAC) (22), corresponding to positions 1406 to 1392 in the 16S ribosomal DNA (rDNA) nucleotide sequence of *Escherichia coli* (7); primer C-complement (GTACACACCGCCCGT; *E. coli* positions 1392 to 1406); primer H (ACACGAGCTGACGACAGCCA; *E. coli* positions 1075 to 1056); primer G (CCAGGGTATCTAATCCTGTT; *E. coli* positions 800 to 781); primer G-complement (AACAGGATTAGATACCTTGG; *E. coli* positions 781 to 800); primer A (GTATTACCGCGG[C/G]TGCTG; *E. coli* positions 536 to 519); primer P (CTGCTGCCTCCCGTAGGAG; *E. coli* positions 357 to 339); primer P-complement (CTACGGGAGGCAGCAG; *E. coli* positions 342 to 357); and primer F₂C (AGAGTTTGATC[A/C]TGGCTC; *E. coli* positions 8 to 25). The resulting sequences were assembled to produce a 1,508-base contiguous rDNA sequence corresponding to *E. coli* positions 15 to 1514. Approximately 85 and 75% of the contiguous sequences of strains TH-22 and TH-23^T, respectively, could be read from more than one primer during assembly. The sequences of strain TH-23^T were obtained from two different cell preparations sequenced at two different times, and the sequences of strain TH-22 were determined at a time different from that of either of the TH-23^T analyses. The overlapping sequences obtained from the two TH-23^T determinations were in agreement.

The assembled 16S rDNA sequences of strains TH-22 and TH-23^T were hand aligned with the equivalent 16S rDNA or rRNA sequences of selected species of eubacteria (see below). The initial sets of prealigned eubacterial sequences were obtained from the Ribosomal Database Project (RDP) (23) and are available via the RDP electronic mail server (version 4.0, updated on 19 June 1994). Each set of aligned sequences was analyzed for maximum parsimony with the program Phylogenetic Analysis Using Parsimony, Macintosh version 3.1.1 (PAUP) (36), in order to construct the most parsimonious phylogenetic tree. Only the phylogenetically informative sites were considered, and alignment gaps were retained in the analysis. A heuristic search was carried out first (with standard program defaults), after which a bootstrap analysis placed confidence limits on the branch points of the resulting phylogenetic trees. Consensus phylogenetic trees for each alignment set were produced by bootstrapping at the greater-than-50% confidence limit, with 100 replications (10).

The phylogenetic position of strains TH-22 and TH-23^T was determined by analyzing the 16S rDNA sequences of these organisms as described above after they were aligned with sets of corresponding sequences for increasingly specific groups of eubacteria. The comparison sequences used for each successive alignment were selected on the basis of the analytical results of the previous alignment. The first alignment included representative species belonging to the 16 major taxonomic groups for which sequences are available in the RDP database (i.e., green sulfur bacteria, spirochetes, purple bacteria, etc.). Analysis of this alignment with PAUP resulted in assignment of strains TH-22 and TH-23^T to the gram-positive phylum (data not shown). Analysis of an alignment that included representative species belonging to the five major subdivisions of the gram-positive phylum then placed TH-22 and TH-23^T in the *Bacillus-Lactobacillus-Streptococcus* subdivision (data not shown), after which analysis of an alignment that included representative species belonging to the 15 subgroups of the *Bacillus-Lactobacillus-Streptococcus* subdivision indicated that strains TH-22 and TH-23^T were most closely related to the "*Bacillus megaterium* group" in the RDP database (data not shown).

After we determined that strains TH-22 and TH-23^T are likely to be most closely related to certain members of the *Bacillus-Lactobacillus-Streptococcus* group as described above, the taxonomic position of these strains within this group was determined more precisely as follows. The 16S rDNA sequences of TH-22 and TH-23^T were hand aligned with the corresponding sequences of 52 selected strains of eubacteria (Table 1). Included in this alignment were the 20 most closely related members of the *Bacillus-Lactobacillus-Streptococcus* group in the RDP database (as determined by levels of sequence similarity and the analyses described above); 13 other, less similar representatives of this group; the 16 thermophilic *Bacillus* species sequenced and analyzed by Rainey et al. (29); two *Clostridium* species; and *Comamonas testosteroni* (formerly *Pseudomonas*

TABLE 1. Levels of similarity between rRNA and rDNA sequences of selected eubacteria and sequences of subsurface isolates TH-22 and TH-23^{Ta}

Taxon (strain[s])	Source of sequence ^b	No. of bases compared	% Sequence similarity to TH-22 and TH-23 ^{Tc}
<i>Alicyclobacillus acidoterrestris</i> (DSM 3923)	RDP	1,464	84.2
<i>Bacillus</i> sp. (starch-negative strain DSM 2349)	EMBL	1,488	92.3
<i>Bacillus alcalophilus</i> (DSM 485; ATCC 27647; NCIMB 10436; NCTC 4553)	RDP	1,327	90.0
<i>Bacillus aminovorans</i> (NCIMB 8292)	RDP	1,493	92.0
<i>Bacillus azotoformans</i> (ATCC 29788)	RDP	1,326	93.2
<i>Bacillus badius</i> (NCDO 1760; ATCC 14574)	RDP	1,324	94.2
<i>Bacillus benzoevorans</i> (NCIB 12555)	RDP	1,328	94.7
<i>Bacillus brevis</i> (NCIB 9372; ATCC 8246)	RDP	1,315	88.3
" <i>Bacillus caldolyticus</i> " (DSM 405)	EMBL	1,486	91.0
" <i>Bacillus caldotenax</i> " (DSM 406)	EMBL	1,489	91.1
" <i>Bacillus caldovelox</i> " (DSM 411)	EMBL	1,491	91.1
<i>Bacillus cereus</i> (NCDO 1771; ATCC 14579; NCTC 2599)	RDP	1,423	92.1
<i>Bacillus circulans</i> (NCDO 1775; ATCC 4513)	RDP	1,326	95.5
<i>Bacillus coagulans</i> (NCDO 1761; DSM 1)	RDP	1,326	93.4
<i>Bacillus cycloheptanicus</i>	RDP	1,461	84.5
<i>Bacillus firmus</i> (NCIB 9366)	RDP	1,327	95.0
" <i>Bacillus flavothermus</i> " (DSM 2641)	EMBL	1,486	92.9
<i>Bacillus kaustophilus</i> (NCIB 8547)	RDP	1,328	90.4
<i>Bacillus larvae</i> (ATCC 9545)	RDP	1,327	87.6
<i>Bacillus lautus</i> (NCIB 12780)	RDP	1,327	92.1
<i>Bacillus lentus</i> (NCDO 1127; ATCC 10840)	RDP	1,328	94.5
" <i>Bacillus maroccanus</i> " (NCIB 10500)	RDP	1,326	93.5
<i>Bacillus megaterium</i> (DSM 32; ATCC 14581; NCDO 1773)	RDP	1,328	93.8
<i>Bacillus methanolicus</i> C1 (NCIMB 13114)	RDP	1,492	95.4
<i>Bacillus pallidus</i> (DSM 3670)	EMBL	1,485	92.2
<i>Bacillus pantothenicus</i> (NCDO 1765; ATCC 14576)	RDP	1,321	91.8
<i>Bacillus polymyxa</i> (NCDO 1774; ATCC 842; DSM 36)	RDP	1,326	87.5
<i>Bacillus psychrophilus</i> W16A (ATCC 23304; DSM 3; IAM 12468; NRS 1530)	RDP	1,430	91.5
<i>Bacillus psychrosaccharolyticus</i> (ATCC 23296)	RDP	1,324	93.5
<i>Bacillus schlegelii</i> (DSM 2000)	EMBL	1,364	82.9
<i>Bacillus simplex</i> (DSM 1321)	RDP	1,328	93.8
<i>Bacillus smithii</i> 1 (DSM 4216)	RDP	1,324	94.0
<i>Bacillus smithii</i> 2 (DSM 4216)	EMBL	1,491	94.2
<i>Bacillus sphaericus</i> 1013 (ATCC 14577; NCDO 1767; NCIMB 9370; NCTC 10338)	RDP	1,329	90.6
<i>Bacillus stearothermophilus</i> (NCDO 1768; ATCC 12980)	RDP	1,328	91.3
<i>Bacillus subtilis</i>	RDP	1,421	92.3
" <i>Bacillus thermoalkalophilus</i> " (DSM 6866)	EMBL	1,484	92.1
<i>Bacillus thermocatenulatus</i> (DSM 730)	EMBL	1,492	91.3
<i>Bacillus thermocloacae</i> (DSM 5250)	EMBL	1,484	90.8
" <i>Bacillus thermodenitrificans</i> " (DSM 465)	EMBL	1,486	91.7
" <i>Bacillus thermodenitrificans</i> " (DSM 466)	EMBL	1,486	91.7
<i>Bacillus thermoglucosidasius</i> (ATCC 43742)	RDP	1,327	92.1
<i>Bacillus thermoleovorans</i> (DSM 5366)	EMBL	1,487	90.9
<i>Bacillus thermoruber</i> (DSM 7064)	EMBL	1,486	89.4
<i>Bacillus thuringiensis</i> (NCIB 9134)	RDP	1,351	91.9
<i>Bacillus tusciae</i> (DSM 2912)	EMBL	1,466	83.0
<i>Clostridium perfringens</i> (ATCC 13124; VPI 5694; NCTC 8237)	RDP	1,430	82.5
<i>Clostridium sporogenes</i> (ATCC 3584; IFO 13950)	RDP	1,452	84.0
<i>Comamonas testosteroni</i> (formerly <i>Pseudomonas testosteroni</i>) RH1104 (ATCC 11996)	RDP	1,455	77.5
" <i>Lactobacillus thermophilus</i> " (ATCC 8317)	RDP	1,430	93.5
<i>Saccharococcus thermophilus</i> (ATCC 43125)	RDP	1,484	93.3
<i>Sporosarcina halophila</i> (NCIMB 2269; ATCC 35676; DSM 2266)	RDP	1,322	91.1

^a The unequivocal portions of the TH-22 and TH-23^T sequences were identical.

^b RDP, Ribosomal Database Project, version 4.0; EMBL, European Molecular Biology Laboratory data bank.

^c Percentage of identical bases for the segments of sequence that could be compared in each case; uncalled bases were counted as matching the bases in the TH-22 and TH-23^T sequence.

testosteroni), which was used as an out-group. The aligned sequences were then analyzed by parsimony and distance matrix methods. The parsimony analysis was performed with PAUP as described above. The distance matrix analysis was carried out by using the PHYLIP package of microcomputer programs (11). Distances were calculated by the method of Jukes and Cantor (20), after which phylogenies were estimated with the FITCH option (in which the Fitch-Margoliash criterion [12] and some related least-squares criteria are used). The following regions were not included in the parsimony and distance matrix analyses because alignment was ambiguous or because there were gaps in some of the

sequences: *E. coli* positions 1 to 28, 71 to 97, 201 to 216, 462 to 470, 840 to 846, 1024 to 1036, and 1432+. Approximately 1,350 bases were used for the analyses. The resulting phylogenetic trees included several clusters of very closely related or nearly identical strains. To clarify the trees for publication, 18 more or less redundant strains that were not closely related to TH-22 and TH-23^T were deleted from the alignment set; one or two representative strains belonging to each cluster were retained in each case. The analyses were then performed again with the smaller set of sequences to produce the trees shown in Fig. 6 and 7.

Analytical methods. Fe(II) contents were measured by the ferrozine method

(34), Mn^{2+} contents were measured by the atomic absorption method, and glucose contents were measured by the hexokinase reaction method (32).

Nucleotide sequence accession numbers. The GenBank database accession numbers for the assembled 16S rDNA sequences are U20384 for the strain TH-22 sequence and U20385 for the strain TH-23^T sequence.

RESULTS AND DISCUSSION

Isolation. Enrichment cultures were prepared by inoculating small (50-mg) pieces of rock into an enrichment medium supplemented with 20 mM formate, 20 mM acetate, and 20 mM MnO_2 as catabolic substrates. Media adjusted to three different pH values (pH 7.2, 8.2, and 9.2) were inoculated, and duplicate preparations of each of these cultures were incubated at 50°C. The enrichment culture at pH 8.2 grew after 40 days of incubation. This culture was serially diluted and inoculated into roll tube medium for isolation. After 3 to 8 weeks of incubation, pinpoint colonies became visible within zones of clearing of the MnO_2 . Colonies were picked, dispersed in MSA

medium containing formate plus acetate, serially diluted, and reinoculated into roll tube medium for purification. Seven strains of MnO_2 -reducing bacteria were isolated and deposited in the U.S. Department of Energy Subsurface Microbial Culture Collection West (SMCC/W) in Portland, Oreg.; these strains included strains TH-17 (= SMCC/W 477), TH-22 (= SMCC/W 478), TH-23^T (= SMCC/W 479^T), and TH-24 (= SMCC/W 480). Strain TH-17 formed colonies after 8 weeks of incubation, but colonies of the other isolates appeared after 3 to 4 weeks. Strain TH-17 differed in other ways from the other six strains and is not described below because it appears not to be a member of the same species.

Morphology. The cells of strains TH-19, TH-20, TH-21, TH-22, TH-23^T, and TH-24 were rod shaped (0.7 to 0.8 μm wide by 4 to 8 μm long). The Gram stain results were ambiguous; the cells appeared to be a darker pink than the gram-negative control cells (*E. coli*). However, thin-section electron micrographs of TH-22 and TH-23^T revealed a gram-positive mor-

FIG. 1. Thin-section electron micrograph of strain TH-23^T, showing the typical gram-positive morphology.

FIG. 2. Effect of temperature on the specific growth rate of strain TH-23^T. Cells were grown on formate plus FeCl₃. Tmin, minimum temperature; Tmax, maximum temperature; Topt, optimum temperature.

phology (Fig. 1). No endospores were seen in electron micrographs, during phase-contrast microscopy, or after malachite green endospore staining. However, the presence of endospores was suggested by the results of studies of oligotrophic survival of strain TH-23^T at high temperatures, as described below. Also, cultures survived heat treatment at 80°C for 10 min. No flagella were seen in electron micrographs, and motility was not observed in wet mounts.

Catabolic substrates. All of the strains grew by oxidizing formate or lactate and reducing MnO₂. They also grew by oxidizing formate and reducing either FeCl₃, trimethylamine oxide, or nitrate, but they could not grow by oxidizing formate and reducing sulfate or thiosulfate. Strains TH-22 and TH-23^T grew anoxically with FeCl₃ as an electron acceptor when formate or lactate was provided as an electron donor but not when the following potential electron donors were provided: acetate, propionate, butyrate, H₂, ethanol, methanol, trimethylamine, Casamino Acids, sucrose, 2-butanol, *n*-pentanol, 2-propanol, and succinate.

Strains TH-22 and TH-23^T grew fermentatively on glucose, producing acetate, lactate, and butyrate. They did not grow (as determined by visual observation and measurement of optical density) or produce acid in MSA medium containing the following substrates: 10 mM sucrose, 10 mM galactose, 10 mM mannose, 10 mM xylose, 10 mM cellobiose, 10 mM arabinose, 10 mM formate, 10 mM acetate, 10 mM propionate, 10 mM

FIG. 3. Effect of pH on the specific growth rate of strain TH-23^T. Cells were grown on formate plus FeCl₃.

FIG. 4. Effect of salinity on the specific growth rate of strain TH-23^T. Cells were grown in MSA medium containing formate, FeCl₃, and various amounts of NaCl.

butyrate, 10 mM malate, 10 mM lactate, 10 mM citrate, 10 mM succinate, 10 mM crotonate, 0.2% (wt/vol) Casamino Acids, 10 mM trimethylamine, 10 mM methanol, 10 mM ethanol, 10 mM 2-propanol, and 10 mM 2-butanol.

Physiology. The strains were strictly anaerobic. None of the six strains was able to grow in MSA medium without reducing agents but with 20 mM formate when air (50 kPa) was added. When cells were inoculated into agar deeps (nutrient agar containing 0.1% glucose), they grew only up to about 5 mm from the surface. The turbidity at the top of the range of growth (i.e., 5 mm below the surface) was not much greater than the turbidity in the depths of the tubes, suggesting that the organisms were not microaerophilic.

All six strains were thermophilic, growing well at 50°C but not at 40 or 65°C. The relationship of the growth of strain TH-23^T to temperature was investigated in more detail. When it was grown at temperatures between 40 and 65°C, strain TH-23^T grew only at temperatures between 45 and 60°C, with the fastest growth occurring at 60°C (Fig. 2). When these data were fitted to the square-root model (30), the estimated growth range was 38.7 to 64.9°C, with the fastest estimated growth occurring at 61.4°C. None of the six strains was strongly alkaliphilic. In MSA medium containing formate and FeCl₃ incubated at 50°C, no growth occurred when the pH was adjusted to 9.2, although growth was as rapid at pH 8.1 as it was at pH 7.0. The effect of pH on growth was investigated in more detail

FIG. 5. Survival of strain TH-23^T under oligotrophic conditions.

FIG. 6. Phylogenetic tree for strains TH-22 and TH-23^T and 34 selected species of eubacteria, based on a distance matrix analysis. The PHYLIP program (11) was used to calculate distances by the method of Jukes and Cantor (20), after which the FITCH option was used to estimate phylogenies from distance matrix data. The eubacterial species used are described in Table 1. *Comamonas testosteroni* was used as the out-group. Abbreviations: C., *Comamonas*; Clos., *Clostridium*; B., *Bacillus*; L., *Lactobacillus*; Sacc., *Saccharococcus*; Alicyclobac., *Alicyclobacillus*.

with strain TH-23^T (Fig. 3). The most rapid growth occurred at pH 7.3. Strain TH-23^T was halotolerant; although it grew fastest in the presence of the lowest levels of salinity tested, it was able to grow in the presence of 2.1 M Na⁺ (Fig. 4).

Cells of TH-22 and TH-23^T could not hydrolyze starch, casein, or gelatin. They produced nitrite from nitrate but did not produce ammonia or N₂ from nitrate.

Oligotrophic survival. The bacteria which we studied were isolated from an environment that is approximately 10⁸ years old, but it is not known whether they or their forebears were incorporated into the sediments at the time that the sediments consolidated or whether they were mobile and colonized the environment at a later time. We tested the ability of strain TH-23^T to survive in a low-nutrient anoxic environment and found that the number of cells decreased rapidly for 1 to 2 days

but that cells that were viable after 2 days remained viable for at least 27 days at 70°C and for at least 52 days at 50°C (Fig. 5). This response to starvation is consistent with hypothesis that most or all vegetative cells died and endospores that either were present in small numbers at the beginning of the experiment or were formed by a minority of the cells during the first 2 days of starvation survived. However, we were not able to verify the presence of endospores microscopically.

Phylogenetic analysis. Corresponding 1,508-base segments of the 16S rRNA genes of subsurface isolates TH-22 and TH-23^T were sequenced and found to be virtually identical. The only discrepancies were discrepancies in a small number of bases that could not be determined unequivocally with the Applied Biosystems model 373A sequencer and were reported

FIG. 7. Consensus phylogenetic tree for strains TH-22 and TH-23^T and 34 selected species of eubacteria, based on a parsimony analysis. The PAUP program (36) was used to analyze approximately 1,350 characteristics of aligned nucleotide sequences for the 36 organisms. The sequences of the eubacterial species were obtained from the RDP and EMBL databases (Table 1). A heuristic search retained two trees with a minimum length of 1,898 steps that differed only in the relative positions of *B. firmus* and *B. lentus*. This tree was generated by bootstrapping at the greater-than-50% confidence limit, with 100 replications (10). The number above each branch is the branch length, and the numbers in circles are the confidence limits for the branch points. *Comamonas testosteroni* was used as the out-group. Abbreviations: *Clos.*, *Clostridium*; *B.*, *Bacillus*; *B. psychrosaccharo.*, *Bacillus psychrosaccharolyticus*; *L.*, *Lactobacillus*; "*B. thermoalkaloph.*," "*Bacillus thermoalkaliphilus*"; *Sacc.*, *Saccharococcus*; "*B. thermodenit.*," "*Bacillus thermodenitrificans*."

as *N.* Thus, the two isolates were virtually identical strains of a single species.

The rDNA sequences described above were aligned with corresponding sequences of members of selected groups of eubacteria and analyzed by the maximum-parsimony method with the PAUP program (36). We found that the sequences of strains TH-22 and TH-23^T were most similar to the sequences of certain species belonging to the *Bacillus-Lactobacillus-Streptococcus* subdivision of the gram-positive phylum of eubacteria. The TH-22 and TH-23^T sequences were then aligned with the sequences of the 20 most closely related species in the RDP database, 13 more distantly related representatives of the *Bacillus-Lactobacillus-Streptococcus* subdivision, 16 thermophilic *Bacillus* species (29), two *Clostridium* species, and *Comamonas testosteroni* (Table 1). A distance matrix analysis of this alignment with the PHYLIP program (11) produced the phylogenetic tree shown in Fig. 6; a maximum-parsimony analysis with PAUP produced the tree shown in Fig. 7.

The distance matrix and parsimony analyses both clearly separated strains TH-22 and TH-23^T from the most closely related members of the *Bacillus-Lactobacillus-Streptococcus* subdivision and clustered them together on a distinct branch.

The most closely related species appeared to be *Bacillus methanolicus*, *B. lentus*, *B. firmus*, *B. circulans*, and *Bacillus azotoformans*. The levels of sequence similarity between the subsurface isolates and the species included in the phylogenetic analyses are shown in Table 1. Most of these levels of similarity were less than 95%; the only exceptions were the levels of similarity for *B. circulans* (95.5%), *B. methanolicus* (95.4%), and *B. firmus* (95.0%).

Taxonomic analysis. The parsimony and distance matrix analyses clearly indicated that strains TH-22 and TH-23^T are members of the genus *Bacillus*. These analyses also clearly distinguished strains TH-22 and TH-23^T from the most closely related members of the genus for which sequences are available; the closest relatives exhibited levels of sequence similarity of about 95%. Levels of sequence similarity of <98% indicate that organisms should be placed in separate species (5, 8). Thus, the new anaerobic strains described in this paper should be classified as members of a new *Bacillus* species. On the basis of our parsimony and distance matrix analysis results we could not determine whether strains TH-22 and TH-23^T are closely related to *Bacillus* strains whose sequences have not been deposited in the RDP and EMBL databases. However, a close relationship between these two anaerobic strains and previously recognized but unsequenced *Bacillus* species is unlikely because there are no previously recognized species that are strictly anaerobic.

Several important *Bacillus* characteristics of four *Bacillus* species that are closely related to strains TH-22 and TH-23^T (i.e., *B. benzoevorans*, *B. circulans*, *B. firmus*, and *B. lentus*) were compared with the characteristics of strains TH-23^T and TH-22; these characteristics included O₂ requirement; production of nitrite from nitrate; and hydrolysis of starch, casein, and gelatin. The results of these comparisons indicated that there are important differences between strains TH-22 and TH-23^T and these four *Bacillus* species. Furthermore, we tested *B. benzoevorans*, *B. circulans*, *B. firmus*, and *B. lentus* for growth on Fe(III) and formate in the absence of O₂, and none of these species was able to grow under these conditions. These phenotypic differences, as well as the phylogenetic distance implied by the results of the 16S rDNA sequences analysis, indicate that strains TH-22 and TH-23^T should be considered members of a separate *Bacillus* species. Assignment of a strictly anaerobic species to the genus *Bacillus* is contrary to the description of the genus, but we propose that this species should be considered an exception to the general description of the genus with respect to O₂ requirements. We propose the new species described below.

Description of *Bacillus infernus*. *Bacillus infernus* (in.fer'nus. M. L. adj. *infernus*, that which comes from below [the ground], named for the deep terrestrial subsurface habitat). Cells are nonmotile rods that are 0.7 to 0.8 by 4 to 8 μm. Endospores not apparent but may be present.

Strictly anaerobic. Thermophilic (61°C), halotolerant (0.6 M Na⁺), and very slightly alkaliphilic (pH 7.3 to 7.8). Growth is fermentative (glucose) or respiratory, with formate and lactate used as electron donors and MnO₂, Fe³⁺, trimethylamine oxide, and nitrate used as electron donors. Nitrate is reduced to nitrite, but nitrate is not further reduced to ammonia or N₂. Neither sulfate nor thiosulfate is reduced.

The habitat is the deep terrestrial subsurface. The type strain is TH-23 (= SMCC/W 479), and reference strains include strains TH-19, TH-20, TH-21, TH-22, and TH-24.

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