Phylogenetic Analysis of Bacteria Preserved in a Permafrost Ice Wedge for 25,000 Years

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Phylogenetic analysis of bacteria preserved within an ice wedge from the Fox Permafrost Tunnel was undertaken by cultivation and molecular techniques. The radiocarbon age of the ice wedge was determined. Our results suggest that the bacteria in the ice wedge adapted to the frozen conditions have survived for 25,000 years.

Ice wedges are wedge-shaped ancient ice (Fig. 1.A) and are among the most common features in permafrost regions, including in northern and central Alaska (7). They grow as a result of repeated cycles of frost cracking follow by the infiltration of snow, meltwater, soil, and other material into the open frost cracks (17). Material incorporated into the ice wedge quickly becomes frozen, and the ice as well as ice in soil and organic particles are thus preserved in a frozen state. The Fox Permafrost Tunnel in Alaska (13), where numerous buried ice wedges are exposed in the tunnel wall (Fig. 1.B), is preserved at a temperature of roughly -3°C by the U.S. Army's Cold Regions Research and Engineering Laboratory. Ice wedges in the tunnel exhibit numerous thin, vertical bands of sediment and ice veinlets characteristic of undisturbed ice wedges (Fig. 1.C), as well as numerous small air bubbles (Fig. 1.D), suggesting that their shapes and fabrics exhibit no signs of thawing (7). If they have not been thawed, it is important to know the age. Microorganisms derived from meltwater and soil particles also might have been trapped and preserved in a frozen state since ice wedge formation. Although there are a number of examples of bacteria in frozen environments (1, 3, 8-12, 19, 21, 24, 25, 30-32, 35-37), no systematic analysis has ever been made on bacteria within a dated ice wedge. Therefore, the objectives of this study

are: to determine the age of the ice wedge sample collected from the Fox Tunnel, to isolate living bacteria, to classify both the isolates and bacterial DNA extracted from the melted ice wedge sample on the basis of the partial 16S rRNA gene sequence, and to examine the temperature sensitivity of ice wedge isolates.

The ice wedge sample was collected from the Fox Permafrost Tunnel (N64°57.084' W147°37.250') and was kept frozen during transportation to the laboratory. The sample was separated into 2 portions. The radiocarbon date and $\delta^{13}C$ as a carbon isotopic ratio of the methane derived from one portion of the ice sample (approximately 2.5 kg) were measured with a Tandetron accelerator mass spectrometry system at Nagoya University. The second portion of the sample (about 50 g) was surface-sterilized by immersing it into a 70% ethanol solution and by burning it to remove the ethanol or contaminated surface ice. We confirmed that the newly exposed surface of ice was not contaminated by stamping it on a cultivation agar and incubating it at 15° C. It was then melted and spread on agar media after aseptical dilution. The used cultivation media were Luria Broth (LB), LBG (LB plus 10 g / l of glucose), R2B (21), a 100-fold diluted LB and LBG, Hickey-tresner revised medium with antibiotics (0.4 g / l of peptone, 0.2 g / l of yeast extract and meat extract, 2.0 g / l of soluble starch, 0.05 g / l of nystatin, 0.01 g / l of cycloheximide, 0.005 g / l of nalidixic acid), minimal medium (MM) (1.0 g / l of K_2HPO_4 and NH_4Cl, 0.2 g / l of $MgSO_4 \cdot 7H_2O$, 0.01 g / l of $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$, 0.1 mg / l of trace elements), MM plus 5.0 g / l of glucose and MME-1, MME-2 (containing 1.0% and 10% of filter-sterilized ice extract obtained from the supernatant of the melted ice wedge, respectively). All media contained 20 g / l of agar and the pH was adjusted to 7.0

with 1N HCl or 1N NaOH. Plates were incubated aerobically at 15° C in the dark for 3 months. Different types of colonies were selected and purified by restreaking on fresh media of the same kind. The partial 16S rRNA genes (*Escherichia coli* positions 27 to 520) were amplified and sequenced from 270 colonies using AmpliTaq PCR Kit and Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). Total nucleic acids were extracted from the precipitates of surface-sterilized melted ice sample using ISOIL (NIPPONGENE). The partial 16S rRNA gene clone library was constructed using pGEM-T Easy vector (Promega). Automatic and manual sequence alignment was performed with the ARB program package (16). A phylogenetic tree was constructed using PHYLIP, version 3.65 (6). The growths of 24 representatives were examined at -5° C, 4° C, 15° C, 27° C and 37° C by measuring diameter of colonies.

The radiocarbon date of 24,884±139 yr BP (data number; NUTA2-3477) was obtained from methane collected from the sampled ice wedge. The stable carbon isotopic ratio was -84.651 ‰, which differs from that of atmospheric methane, demonstrating that any contamination by atmospheric air was negligible. Bacterial colonies grew at concentrations from 10^5 up to 10^6 colony-forming units (CFU) per ml of melted ice. In total, 270 aerobic or facultatively anaerobic bacteria were isolated. Most of the isolates were non-spore-forming bacteria. When the sequences with greater than 98% similarity were treated as the same species, isolates and 273 clonal types were grouped into 41 OTUs and 12 OTUs, respectively. The number of OTUs was determined by DOTUR program (http://www.plantpath.wisc.edu/fac/joh/dotur.html) (28). A phylogenetic tree between representatives of OTUs and their closest relatives was constructed with distance data by a

neighbour-joining method (26). Bootstrap analyses for 1,000 replicates were performed. Both OTUs of the isolates and clones were affiliated with three different classes of *Actinobacteria*, *Bacilli* and *Gammaproteobacteria* (Fig. 2). Similar topologies of the OTUs were observed from the trees generated with the maximum-likelihood and maximum-parsimony methods (data not shown). The 36 OTUs of isolates and 4 clonal OTUs were affiliated with the order of *Actinomycetales* and were closely related to the genera such as *Arthrobacter*, *Brachybacterium*, *Cryobacterium*, *Microbacterium* and *Rhodococcus*. In the *Bacilli* branch, 4 OTUs of isolates and 3 OTUs of clones were closely related to the genera *Planococcus* and *Carnobacterium*. The dominant taxon of clones was *Gammaproteobacteria* (93.1% of the total number of clones). In this class, isolates and clones were closely related to *Lysobacter* and *Pseudomonas*, respectively. Actually, the strains which were identical to the representative clonal type no. 206 in 16S rRNA gene sequences had been isolated mainly from MME-2 agar plates, however, unfortunately, all of these isolates could not be subcultured. All of the isolates that were examined for their sensitivity to temperature grew at 4° C and 20° C, but not 37° C. Ten isolates which were closely related to the genera *Arthrobacter*, *Planococcus*, *Microbacterium* and *Rhodococcus* could grow at -5° C after 3 months cultivation (Fig. 2).

The results demonstrate that the Fox tunnel ice wedge has remained continuously frozen for the past 25,000 years. From the ice we collected, living bacteria were reproduced at concentrations as high as 10^6 CFU per ml of melted ice. Although bacteria are reported to be rarely recovered from ice wedges (8, 10), this study clearly demonstrates the existence of viable bacteria within the ice wedge ice. We could easily recognize soil particles in the

ice wedge sample melt, suggesting that these suspended solids might be a habitat that protected cells from ice crystals. The bacteria isolated from Siberian permafrost on an LB medium were affiliated with *Actinobacteria*, *Bacilli*, *Alpha*, *Beta* and *Gammaproteobacteria* (31). On the contrary, *Proteobacteria* were not isolated from our ice wedge sample on the same medium (data not shown), indicating that the higher taxonomic levels of the ice wedge isolates were less diverse. This is consistent with the results of molecular analysis. No clonal type affiliated with *Alpha* and *Betaproteobacteria* appeared in the clone library. In general, the bacterial community can be distorted by several biases such as, DNA extraction (23), or PCR (33). However, phylogenetic diversity among the 16S rRNA gene clones was considered to be remarkably low. To assess if the number of screened clones was sufficient for an estimation of diversity in the clone library, rarefaction analysis was performed by DOTUR program. The expected number of the OTUs was plotted against the number of clones in various distance level. The calculated rarefactions curves of clonal OTUs nearly reached to an asymptote at a distance level above 1%, indicating that the screened number of clones was enough (Fig. S1 in the supplemental material). On the basis of the results that some of these ice wedge isolates were able to grow at -5° C, i.e., at the *in situ* temperature, we assumed that these bacteria accomplished better strategies for surviving in the ice wedge. Similarly, *Psychrobacter* sp. isolated from a Siberian permafrost cryopeg was reported to grow at -10° C, the temperature of cryopegs (1). Although it is still unknown whether they are active or dormant *in situ*, these results suggest that bacteria that were adapted to ice wedge conditions have survived for thousands of years. Our investigation of these adapted bacteria not only provides novel information

about adaptation or survival mechanisms under extreme conditions but also may lead to a wide variety of biotechnological applications that had not previously been explored.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the representative isolates and clones reported in this study were deposited in GenBank under accession numbers AB272756 to AB272838.

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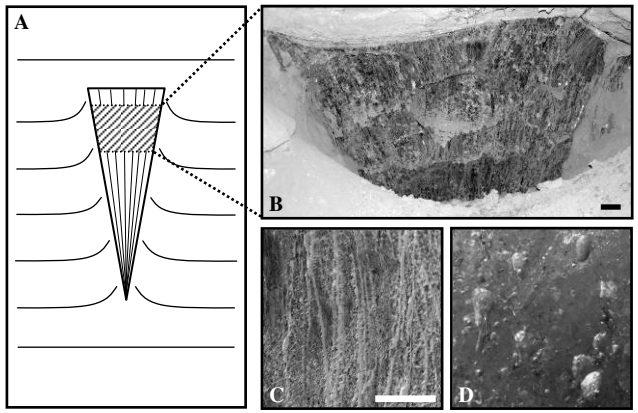
Figure legends

FIG. 1. Fabrics of the ice wedge in the Fox Permafrost Tunnel. Each scale-bar indicates 0.1 m. (A) Schematic pattern of ice wedge in permafrost. (B) Exposed part of the ice wedge. (C) Foliation of ice indicating annual veinlets. (D) Air bubbles in the ice (1-2 mm in diameter).

FIG. 2. Phylogenetic relationship of the representative isolates, clonal types (bold) and their closest relatives based on partial 16S rRNA gene sequences. Bootstrap values that were above 50% are shown at the nodes. The scale-bar represents 1 substitution per 10 nucleotides. *Escherichia coli* (X80725) was used as the outgroup. Asterisks and shaded

clusters indicate the representative isolates that were examined to their sensitivity to temperature and those that grew at -5° C, respectively.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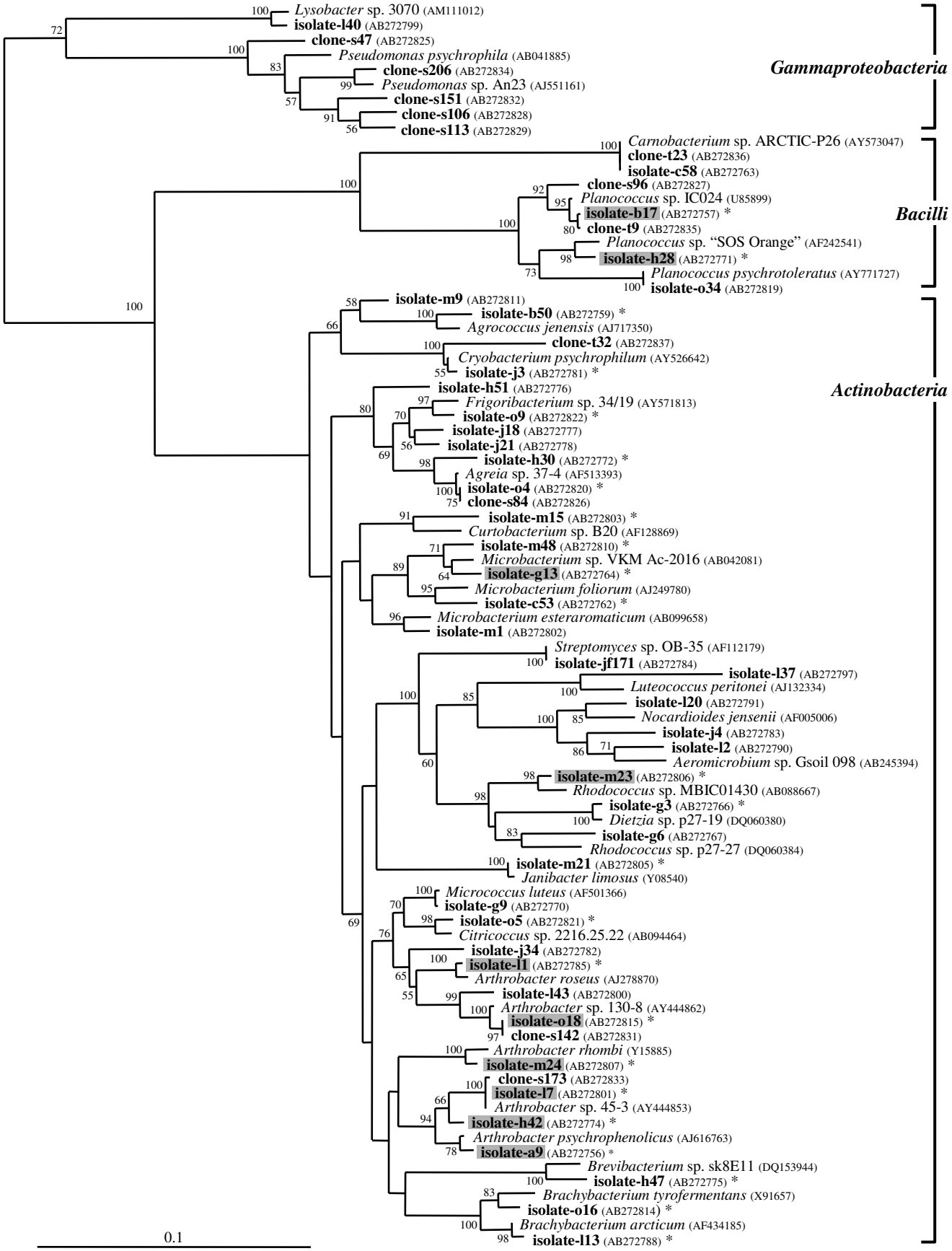
Gammaproteobacteria
Bacilli
Actinobacteria
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clone-s47 (AB272825)
Pseudomonas psychrophila (AB041885)
clone-s206 (AB272834)
Pseudomonas sp. An23 (AJ551161)
clone-s151 (AB272832)
clone-s106 (AB272828)
clone-s113 (AB272829)
Carnobacterium sp. ARCTIC-P26 (AY573047)
clone-t23 (AB272836)
isolate-c58 (AB272763)
clone-s96 (AB272827)
Planococcus sp. IC024 (U85899)
isolate-b17 (AB272757) *
clone-t9 (AB272835)
Planococcus sp. "SOS Orange" (AF242541)
isolate-h28 (AB272771) *
Planococcus psychrotoleratus (AY771727)
isolate-o34 (AB272819)
isolate-m9 (AB272811)
isolate-b50 (AB272759) *
Agrococcus jenensis (AJ717350)
clone-t32 (AB272837)
Cryobacterium psychrophilum (AY526642)
isolate-j3 (AB272781) *
isolate-h51 (AB272776)
Frigoribacterium sp. 34/19 (AY571813)
isolate-o9 (AB272822) *
isolate-j18 (AB272777)
isolate-j21 (AB272778)
isolate-h30 (AB272772) *
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isolate-o4 (AB272820) *
clone-s84 (AB272826)
isolate-m15 (AB272803) *
Curtobacterium sp. B20 (AF128869)
isolate-m48 (AB272810) *
Microbacterium sp. VKM Ac-2016 (AB042081)
isolate-g13 (AB272764) *
Microbacterium foliorum (AJ249780)
isolate-c53 (AB272762) *
Microbacterium esteraromaticum (AB099658)
isolate-m1 (AB272802)
Streptomyces sp. OB-35 (AF112179)
isolate-jf171 (AB272784)
isolate-l37 (AB272797)
Luteococcus peritonei (AJ132334)
isolate-l20 (AB272791)
Nocardioides jensenii (AF005006)
isolate-j4 (AB272783)
isolate-l2 (AB272790)
Aeromicrobium sp. Gsoil 098 (AB245394)
isolate-m23 (AB272806) *
Rhodococcus sp. MBIC01430 (AB088667)
isolate-g3 (AB272766) *
Dietzia sp. p27-19 (DQ060380)
isolate-g6 (AB272767)
Rhodococcus sp. p27-27 (DQ060384)
isolate-m21 (AB272805) *
Janibacter limosus (Y08540)
Micrococcus luteus (AF501366)
isolate-g9 (AB272770)
isolate-o5 (AB272821) *
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isolate-j34 (AB272782)
isolate-l1 (AB272785) *
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isolate-l43 (AB272800)
Arthrobacter sp. 130-8 (AY444862)
isolate-o18 (AB272815) *
clone-s142 (AB272831)
Arthrobacter rhombi (Y15885)
isolate-m24 (AB272807) *
clone-s173 (AB272833)
isolate-l7 (AB272801) *
Arthrobacter sp. 45-3 (AY444853)
isolate-h42 (AB272774) *
Arthrobacter psychrophenolicus (AJ616763)
isolate-a9 (AB272756) *
Brevibacterium sp. sk8E11 (DQ153944)
isolate-h47 (AB272775) *
Brachybacterium tyrofermentans (X91657)
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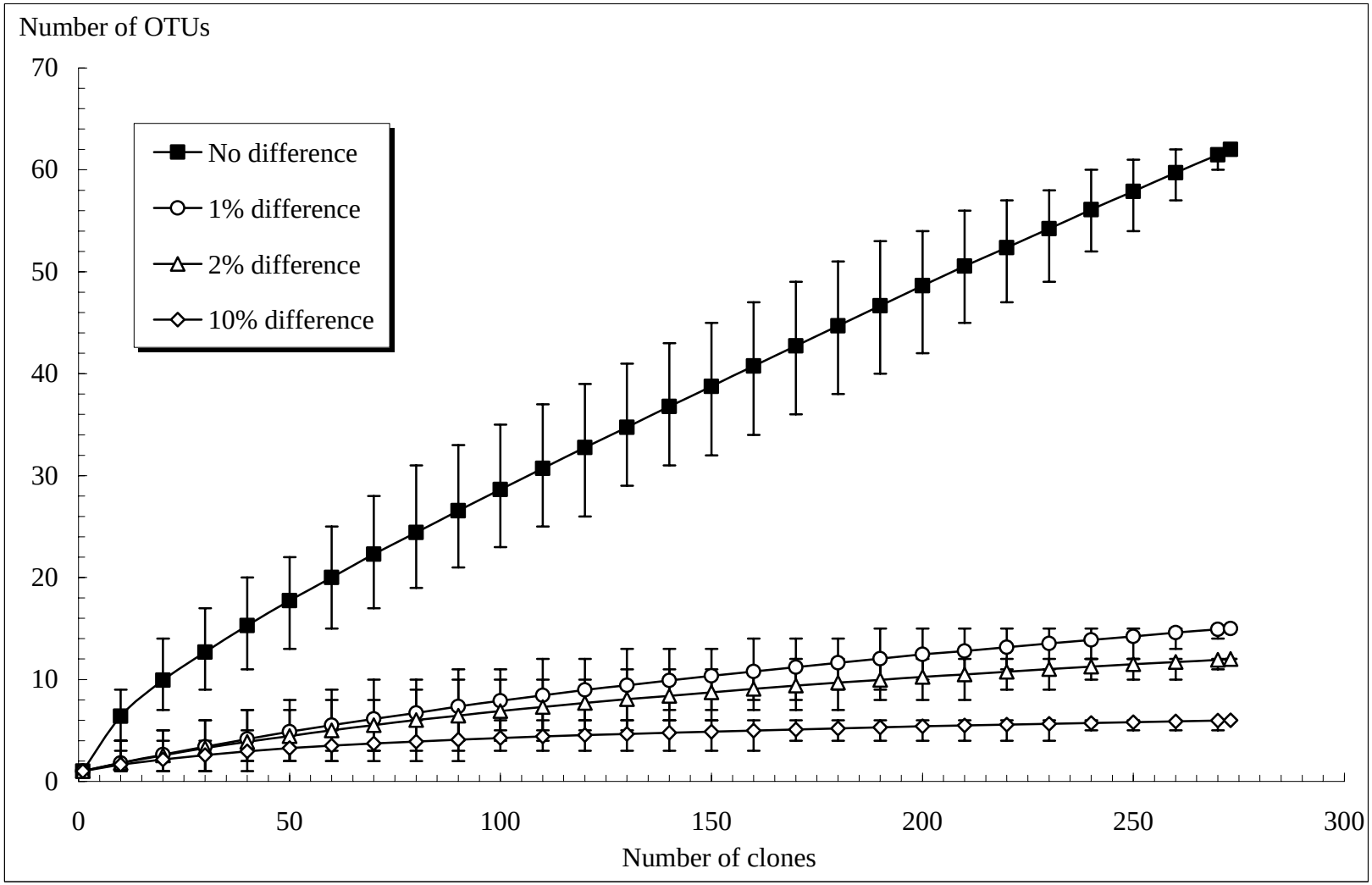

FIG. S1. Rarefaction curves for OTUs at given distance levels of partial 16S rRNA gene clone library. Error bars represent the 95% confidence intervals.