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

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

6
7 Ice wedges are wedge-shaped ancient ice (Fig. 1.A) and are among the most
8 common features in permafrost regions, including in northern and central Alaska (7). They
9 grow as a result of repeated cycles of frost cracking follow by the infiltration of snow,
10 meltwater, soil, and other material into the open frost cracks (17). Material incorporated
11 into the ice wedge quickly becomes frozen, and the ice as well as ice in soil and organic
12 particles are thus preserved in a frozen state. The Fox Permafrost Tunnel in Alaska (13),
13 where numerous buried ice wedges are exposed in the tunnel wall (Fig. 1.B), is preserved at
14 a temperature of roughly -3°C by the U.S. Army's Cold Regions Research and Engineering
15 Laboratory. Ice wedges in the tunnel exhibit numerous thin, vertical bands of sediment and
16 ice veinlets characteristic of undisturbed ice wedges (Fig. 1.C), as well as numerous small
17 air bubbles (Fig. 1.D), suggesting that their shapes and fabrics exhibit no signs of thawing
18 (7). If they have not been thawed, it is important to know the age. Microorganisms derived
19 from meltwater and soil particles also might have been trapped and preserved in a frozen
20 state since ice wedge formation. Although there are a number of examples of bacteria in
21 frozen environments (1, 3, 8-12, 19, 21, 24, 25, 30-32, 35-37), no systematic analysis has
22 ever been made on bacteria within a dated ice wedge. Therefore, the objectives of this study

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

5 The ice wedge sample was collected from the Fox Permafrost Tunnel
6 (N64°57.084' W147°37.250') and was kept frozen during transportation to the laboratory.
7 The sample was separated into 2 portions. The radiocarbon date and $\delta^{13}\text{C}$ as a carbon
8 isotopic ratio of the methane derived from one portion of the ice sample (approximately 2.5
9 kg) were measured with a Tandetron accelerator mass spectrometry system at Nagoya
10 University. The second portion of the sample (about 50 g) was surface-sterilized by
11 immersing it into a 70% ethanol solution and by burning it to remove the ethanol or
12 contaminated surface ice. We confirmed that the newly exposed surface of ice was not
13 contaminated by stamping it on a cultivation agar and incubating it at 15° C. It was then
14 melted and spread on agar media after aseptical dilution. The used cultivation media were
15 Luria Broth (LB), LBG (LB plus 10 g / l of glucose), R2B (21), a 100-fold diluted LB and
16 LBG, Hickey-tresner revised medium with antibiotics (0.4 g / l of peptone, 0.2 g / l of yeast
17 extract and meat extract, 2.0 g / l of soluble starch, 0.05 g / l of nystatin, 0.01 g / l of
18 cycloheximide, 0.005 g / l of nalidixic acid), minimal medium (MM) (1.0 g / l of K_2HPO_4
19 and NH_4Cl , 0.2 g / l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g / l of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1
20 mg / l of trace elements), MM plus 5.0 g / l of glucose and MME-1, MME-2 (containing
21 1.0% and 10% of filter-sterilized ice extract obtained from the supernatant of the melted ice
22 wedge, respectively). All media contained 20 g / l of agar and the pH was adjusted to 7.0

1 with 1N HCl or 1N NaOH. Plates were incubated aerobically at 15° C in the dark for 3
2 months. Different types of colonies were selected and purified by restreaking on fresh
3 media of the same kind. The partial 16S rRNA genes (*Escherichia coli* positions 27 to 520)
4 were amplified and sequenced from 270 colonies using AmpliTaq PCR Kit and Big Dye
5 Terminator cycle sequencing ready reaction kit (Applied Biosystems). Total nucleic acids
6 were extracted from the precipitates of surface-sterilized melted ice sample using ISOIL
7 (NIPPONGENE). The partial 16S rRNA gene clone library was constructed using pGEM-T
8 Easy vector (Promega). Automatic and manual sequence alignment was performed with the
9 ARB program package (16). A phylogenetic tree was constructed using PHYLIP, version
10 3.65 (6). The growths of 24 representatives were examined at -5° C, 4° C, 15° C, 27° C and
11 37° C by measuring diameter of colonies.

12 The radiocarbon date of $24,884 \pm 139$ yr BP (data number; NUTA2-3477) was obtained
13 from methane collected from the sampled ice wedge. The stable carbon isotopic ratio was
14 -84.651 ‰, which differs from that of atmospheric methane, demonstrating that any
15 contamination by atmospheric air was negligible. Bacterial colonies grew at concentrations
16 from 10^5 up to 10^6 colony-forming units (CFU) per ml of melted ice. In total, 270 aerobic
17 or facultatively anaerobic bacteria were isolated. Most of the isolates were
18 non-spore-forming bacteria. When the sequences with greater than 98% similarity were
19 treated as the same species, isolates and 273 clonal types were grouped into 41 OTUs and
20 12 OTUs, respectively. The number of OTUs was determined by DOTUR program
21 (<http://www.plantpath.wisc.edu/fac/joh/dotur.html>) (28). A phylogenetic tree between
22 representatives of OTUs and their closest relatives was constructed with distance data by a

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

18 The results demonstrate that the Fox tunnel ice wedge has remained continuously
19 frozen for the past 25,000 years. From the ice we collected, living bacteria were reproduced
20 at concentrations as high as 10⁶ CFU per ml of melted ice. Although bacteria are reported to
21 be rarely recovered from ice wedges (8, 10), this study clearly demonstrates the existence
22 of viable bacteria within the ice wedge ice. We could easily recognize soil particles in the

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

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

3
4 **Nucleotide sequence accession numbers.** The 16S rRNA gene sequences of the
5 representative isolates and clones reported in this study were deposited in GenBank under
6 accession numbers AB272756 to AB272838.

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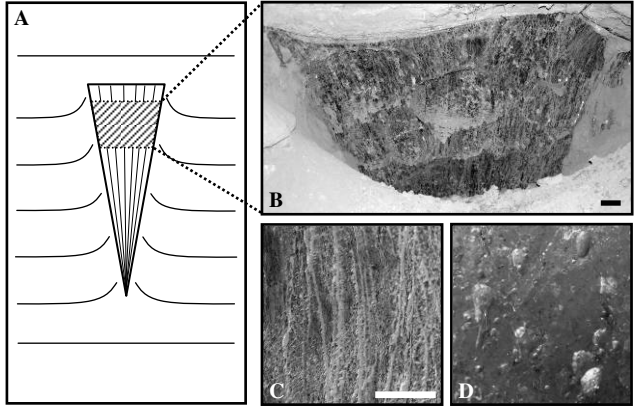
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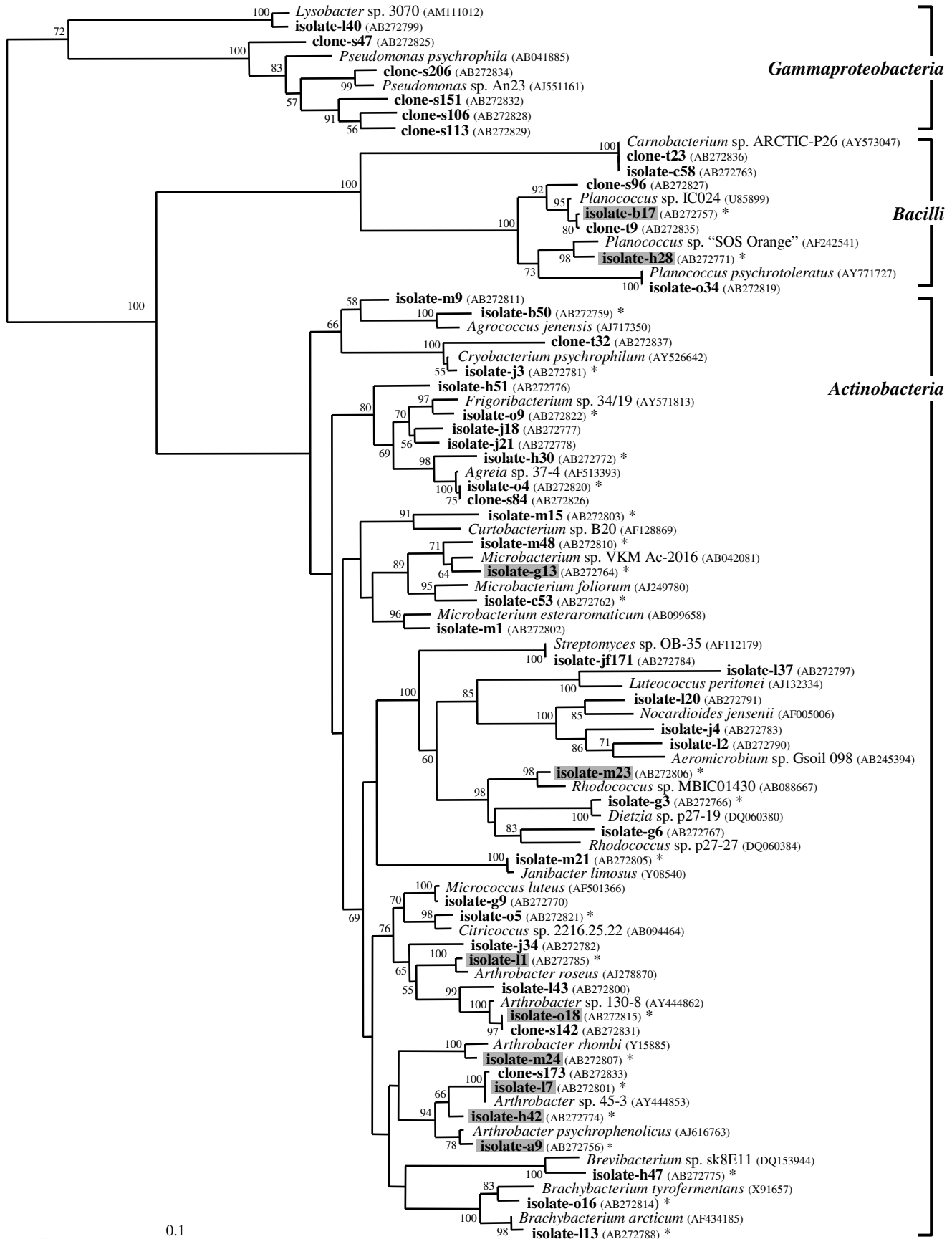
14 FIG. 1. Fabrics of the ice wedge in the Fox Permafrost Tunnel. Each scale-bar indicates 0.1
15 m. (A) Schematic pattern of ice wedge in permafrost. (B) Exposed part of the ice wedge.
16 (C) Foliation of ice indicating annual veinlets. (D) Air bubbles in the ice (1-2 mm in
17 diameter).

18

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

- 1 clusters indicate the representative isolates that were examined to their sensitivity to
- 2 temperature and those that grew at -5°C , respectively.





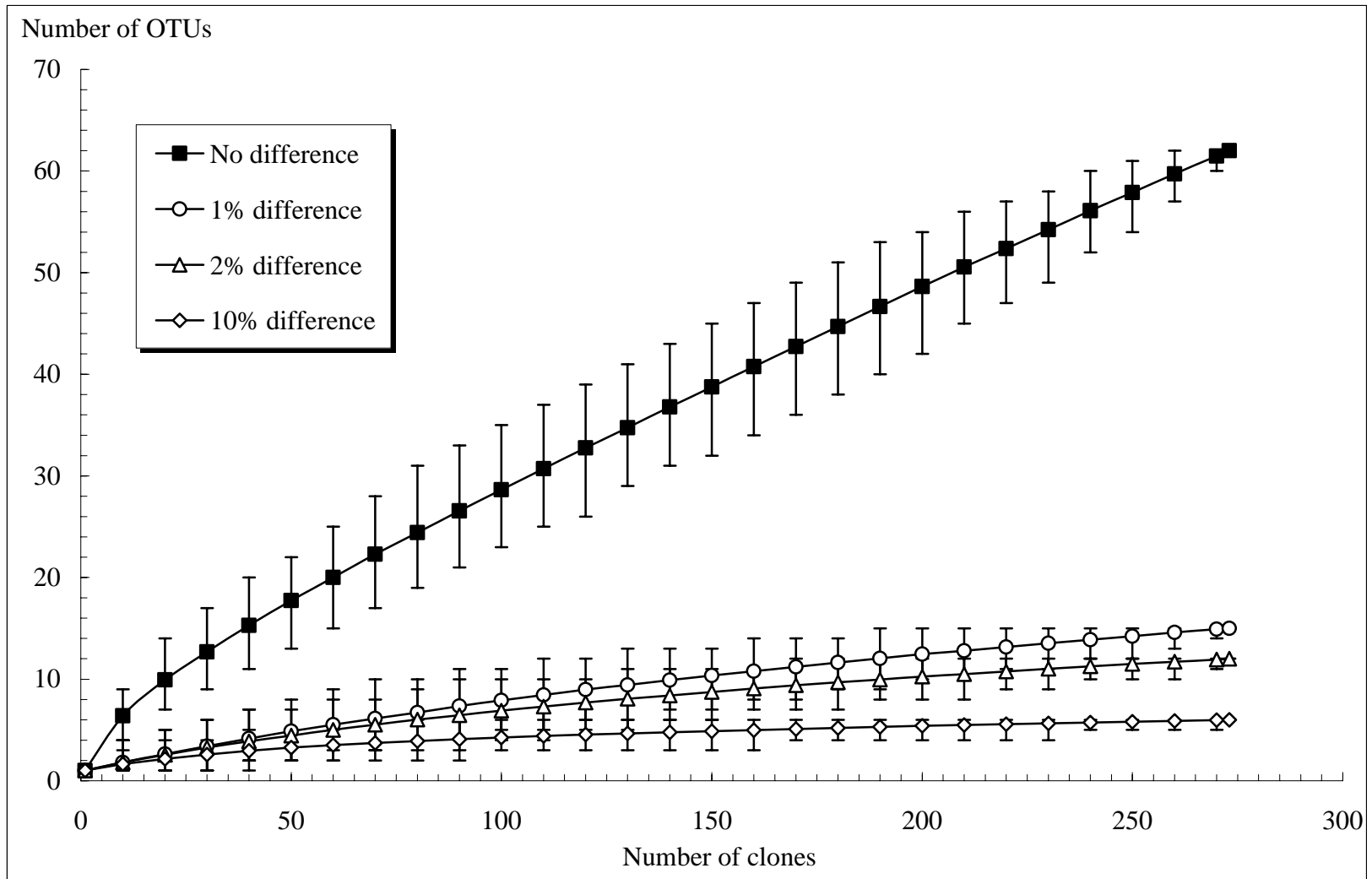


FIG. S1. Rarefaction curves for OTUs at given distance levels of partial 16S rRNA gene clone library. Error bars represent the 95% confidence intervals.