

## Identification of Bacterial Isolates Obtained from Intestinal Contents Associated with 12,000-Year-Old Mastodon Remains

A. N. RHODES,<sup>1</sup> J. W. URBANCE,<sup>1</sup> H. YOUNG,<sup>1</sup> H. CORLEW-NEWMAN,<sup>1</sup> C. A. REDDY,<sup>1</sup> M. J. KLUG,<sup>1</sup>  
J. M. TIEDJE,<sup>1\*</sup> AND D. C. FISHER<sup>2</sup>

NSF Center for Microbial Ecology and Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101,<sup>1</sup> and Museum of Paleontology, University of Michigan, Ann Arbor, Michigan 48109-1079<sup>2</sup>

Received 7 July 1997/Accepted 2 December 1997

**Mastodon (*Mammot americanum*) remains unearthed during excavation of ancient sediments usually consist only of skeletal material, due to postmortem decomposition of soft tissues by microorganisms. Two recent excavations of skeletal remains in anoxic sediments in Ohio and Michigan, however, have uncovered organic masses which appear to be remnants of the small and large intestines, respectively. Macrobotanical examinations of the composition of these masses revealed assemblages of plant material radiocarbon dated to approximately 11,500 years before the present and thought to be incompletely digested food remains from this extinct mammal. We attempted to cultivate and identify bacteria from the intestinal contents, bone-associated sediments, and sediments not in proximity to the remains using a variety of general and selective media. In all, 295 isolates were cultivated, and 38 individual taxa were identified by fatty acid-methyl ester (FAME) profiles and biochemical characteristics (API-20E). The taxonomic positions of selected enteric and obligately anaerobic bacteria were confirmed by 16S ribosomal DNA (rDNA) sequencing. Results indicate that the intestinal and bone-associated samples contained the greatest diversity of bacterial taxa and that members of the family *Enterobacteriaceae* represented 41% of all isolates and were predominant in the intestinal masses and sediments in proximity to the skeleton but were uncommon in the background sediments. *Enterobacter cloacae* was the most commonly identified isolate, and partial rDNA sequencing revealed that *Rahnella aquatilis* was the correct identity of strains suggested by FAME profiles to be *Yersinia enterocolitica*. No *Bacteroides* spp. or expected intestinal anaerobes were recovered. The only obligate anaerobes recovered were clostridia, and these were not recovered from the small intestinal masses. Microbiological evidence from this study supports other, macrobotanical data indicating the intestinal origin of these masses. Whether these organisms are direct descendants of the original intestinal microbiota, however, cannot be established.**

An opportunity to examine the intestinal contents of an extinct mammal rarely presents itself to science. Consumption of soft tissues by predators and scavengers or rapid postmortem decomposition by microorganisms usually results in dispersion and mixing of intestinal contents with materials from the environment, thereby making subsequent analyses of limited scientific value. However, under appropriate physical and chemical conditions, intestinal contents may be preserved in a relatively intact state (13). Recent excavations at two separate sites disclosed masses of plant materials preserved in late-Pleistocene pond sediments and associated with skeletal remains of American mastodons (*Mammot americanum*). These sites are referred to below as Burning Tree (Licking County, Ohio) and Heisler (Calhoun County, Michigan). The plant materials from these sites varied in degree of comminution but were consistently distinct in texture and color from surrounding sediment and resembled plant materials from the intestinal tract of modern herbivores (Fig. 1). The size and shape of the original masses were compatible with the intestinal dimensions of modern elephants. Based on these physical criteria, the plant materials were provisionally identified as intestinal contents.

The sediments in which these plant materials were preserved apparently remained undisturbed within a continuously wet, cool, low-pH, anoxic environment since the original deposition

at each site. The putative intestinal mass at Burning Tree occurred in peat near an articulated series of ribs and vertebrae. This mass was cylindrical, about 12 cm in diameter and 60 cm in length, suggesting attribution to the small intestine of the mastodon (10). A slight constriction separated this cylinder into "primary" and "secondary" masses with no indication of proximodistal polarity. Macrobotanical analysis demonstrated that the plant assemblage of the cylindrical mass differed significantly from that of the surrounding sediment, implying a different mechanism or circumstance of accumulation. Seeds in the cylindrical mass were restricted to plant species that set seed in autumn (10), implying a probable time of death for the mastodon during this part of the year. This same season of death was independently established through analysis of tusk growth lines, corroborating the provisional identification of plant materials as intestinal contents (4). Radiocarbon dating also supported this interpretation. Deciduous twigs from the Burning Tree mass were dated (10) at  $11,660 \pm 120$  years before present (BP) (Beta-38241/ETH-6758) and  $11,450 \pm 70$  years BP (Pitt-0832), and bone collagen (XAD-purified gelatin hydrolysate) was dated (5) at  $11,390 \pm 80$  years BP (NSRL-283/AA-6980).

At the Heisler site, the putative intestinal contents consisted of ovoidal masses of sand and gravel (maximum diameter, ca. 30 cm) with a peripheral zone of comminuted plant debris. These ovoidal masses occurred along with bones in a peaty marl and were interpreted as "clastic anchors" made by prehistoric humans (Fig. 2). Such anchors were apparently used to help hold mastodon carcass parts on the pond bottom, or keep them tethered to a chosen area in the pond, as a primitive but

\* Corresponding author. Mailing address: Center for Microbial Ecology, Michigan State University, 540 Plant and Soil Sciences Bldg., East Lansing, MI 48823-1101. Phone: (517) 353-9021. Fax: (517) 353-2917. E-mail: tiedje@pilot.msu.edu.

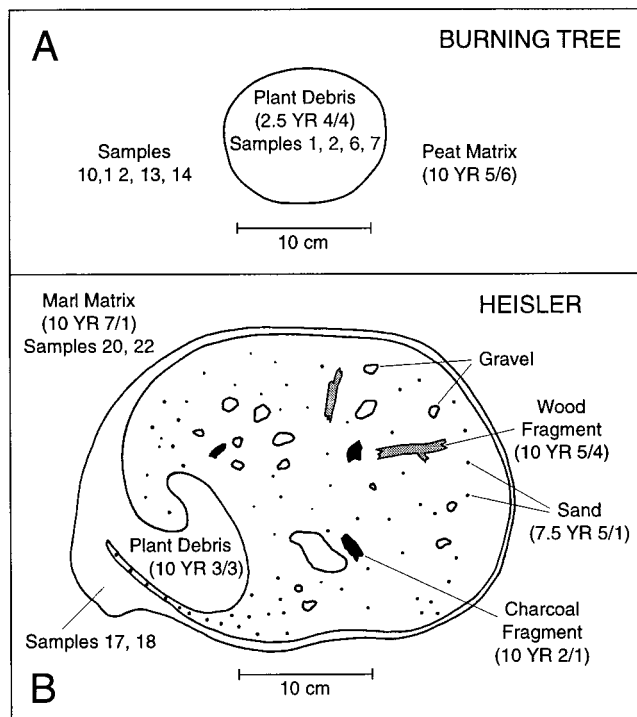


FIG. 1. Schematic vertical cross sections through features inferred as preserved intestinal contents (Plant Debris) at the Burning Tree (A) mastodon site (Licking Co., Ohio) and at the Heisler (B) mastodon site (Calhoun Co., Mich.). The matrix and major constituents of each feature are labelled and Munsell soil color codes are given for all components except gravel. Sample numbers (cited also in the text and tables) are shown in representative locations.

effective strategy for winter storage of meat (2, 3). Hypothetically, these anchors were formed by filling short lengths of mastodon intestine with sand and gravel. Judging from their size, the anchors were presumably made from segments of large intestine. The comminuted plant material consisted of chewed and partly digested material that apparently lined the



FIG. 2. Photograph of a block of material cut at right angles and removed from the margin of one of the sand-filled intestinal features at the Heisler mastodon site (Calhoun Co., Mich.). The narrow, curved, dark zone evident on the strongly illuminated face of the block is comminuted plant debris inferred to be original intestinal contents. Such material was the source of samples 17 and 18 from the Heisler site. Granular material enclosed by the zone of plant debris is sand, inferred to have been introduced by human activity. Diameter of trowel handle (left background) is ca. 2.5 cm.

walls of the large intestine at the time sediments were introduced into the intestinal lumen. Pollen analysis (15) of putative intestinal contents from the Heisler site confirmed their distinctiveness from surrounding sediment and also indicated autumn as the time of death. The latter was confirmed by tusk growth line analysis (2). Again, radiocarbon dates supported this association (3), as plant debris from a Heisler clastic anchor was dated at  $11,380 \pm 130$  years BP (Beta-39043) and bone collagen (XAD-purified gelatin hydrolysate) gave a date of  $11,770 \pm 110$  years BP (NSRL-282/AA-6979).

To evaluate more definitively the hypothesis of intestinal origin for these unusual assemblages of plant material, we conducted microbiological analyses to determine whether facultatively anaerobic gram-negative rods or obligately anaerobic organisms, commonly found in mammalian intestinal tracts, were associated with these materials. Initial investigations identified *Enterobacter cloacae* from masses collected at the Burning Tree site but not from background controls (10). We have expanded on this earlier effort by analyzing a larger number of samples, adding more rigorous controls, and applying more refined methods to characterize the bacteria present. Our results indicate that numerous facultatively anaerobic gram-negative rods representing genera of the family *Enterobacteriaceae* are prevalent in the presumed intestinal masses from the Burning Tree site. Enteric organisms are also common in samples associated with Burning Tree skeletal remains. Obligately anaerobic clostridia of potential intestinal origin were more prevalent in clastic anchors from Heisler; however, a clear microbiological distinction between clastic anchors and background sediments was not observed in this study.

#### MATERIALS AND METHODS

**Sample design.** Previous field sampling collected the following sets of samples: (i) multiple portions of the putative intestinal contents from each site, (ii) sediment closely associated with bone but not directly associated with the intestinal contents (Burning Tree site only), (iii) background sediment located immediately adjacent to a putative intestinal mass (Heisler site only), and (iv) background sediment located some distance from both bone and intestinal contents. The second category of samples represents one type of control, namely carcass parts that presumably once included both bone and soft tissues that would have been attached to the bone. Although such carcass parts could have been contaminated by intestinal contents during butchering (and therefore prior to introduction into the pond environment), they showed no macroscopic traces of plant residue at the time of excavation. The purpose in examining these samples was to determine whether carcass units not associated with intestinal materials developed microbial associations similar to those observed for putative intestinal contents. This might have occurred, for instance, if the microorganisms colonized carcass units and intestinal masses sometime after deposition in the pond sediment. The third category of samples represents sediment deposited nearly contemporaneous but not associated with the mastodon remains. These samples retain sedimentological distinctness while maximizing proximity to putative intestinal contents. The fourth category of samples represents microflora occurring in the pond sediments independent of mastodon remains. These samples were derived from stratigraphic levels above (postdating), at (contemporaneous with), and below (predating) the level at which mastodon remains were preserved.

Samples were collected aseptically in the field, as a coherent mass, and were frozen at  $-20^{\circ}\text{C}$  within 10 h following collection. Subsamples were later prepared by briefly removing the original samples from the freezer and aseptically fracturing them. Subsamples consisted of 1- to 3-g fragments selected from the interior of the original samples. These fragments were placed into sterile airtight containers and stored at  $-20^{\circ}\text{C}$  until analysis. In a few cases, original samples experienced freeze-thaw cycles prior to microbiological analysis. When possible, only samples without a history of freeze-thawing were chosen for analysis. This resulted in elimination of the third sample category described above; however, samples from all other categories were analyzed. Table 1 contains the identification numbers, origins, pHs, and brief descriptions of subsamples used for this study.

**Media and reagents.** Unless otherwise specified, all media were obtained commercially and prepared following recommendations of the manufacturer. For aerobic enrichments and isolations the following media were used: brain heart infusion (BHI) liquid and agar, MacConkey agar, brilliant green agar, and eosin-methylene blue (EMB) agar. Prereduced, anaerobically sterilized (PRAS) BHI, Sweet-E (SE), chopped meat-glucose, and peptone-yeast extract-glucose

TABLE 1. Locations and characteristics of samples collected from mastodon sites in Ohio and Michigan<sup>a</sup>

Sample no. and parameter	Description and origin	pH	TOC <sup>b</sup> (%)	AODC <sup>c</sup>	MPN <sup>d</sup>	
					Aer.	Ana.
<b>Burning Tree</b>						
1	Primary intestinal mass, central region	6.1	45.1 <sup>e</sup>	8.83	7.73	6.31
2	Primary intestinal mass, central region, 10 cm from sample 1	ND <sup>f</sup>	ND	9.02	ND	ND
6	Primary intestinal mass, end opposite sample 7	6.6	23.3	9.39	7.32	4.89
7	Secondary intestinal mass, end opposite sample 6	ND	50.2	9.71	7.26	3.80
Avg (SD)				9.24 (0.39)	7.44 (0.26)	5.00 (1.26)
4	Rib sediment, adjacent to bone and ca. 1 m from intestinal mass (same stratum)	6.6	ND	8.46	4.07	3.90
8	Limb sediment, adjacent to bone and >2 m from intestinal mass (same stratum)	ND	22.4	9.27	4.35	3.63
Avg (SD)				8.87 (0.57)	4.21 (0.20)	3.77 (0.19)
10	Sediment, stratum I (below mastodon, >3 m distant horizontally)	ND	ND	7.25	5.16	3.20
12	Sediment, stratum IIIA (below mastodon), >3 m distant horizontally	ND	ND	8.79	3.00	3.52
13	Sediment, stratum IIIB (same stratum as mastodon), >3 m distant horizontally	6.8	24.4	ND	ND	ND
14	Sediment, stratum IIIC (above mastodon), >3 m distant horizontally	ND	38.3	8.69	— <sup>g</sup>	—
Avg (SD)				8.24 (0.86)	4.08 (1.53)	3.36 (0.23)
<b>Heisler</b>						
17	Clastic anchor, peripheral zone of plant debris well developed	ND	12.9	9.07	6.69	4.25
18	Clastic anchor, peripheral zone of plant debris present in traces only	ND	ND	7.05	6.41	4.49
Avg (SD)				8.06 (1.43)	6.55 (0.20)	4.37 (0.17)
20	Sediment, stratigraphically 20 cm above mastodon remains, 1 to 2 m distant horizontally	ND	ND	7.13	4.10	3.72
22	Sediment, stratigraphically 15 cm below mastodon remains, 1 to 2 m distant horizontally	ND	11.7	8.31	5.33	2.33
Avg (SD)				7.72 (0.83)	4.42 (0.87)	3.03 (0.98)

<sup>a</sup> Numbers of bacteria in Burning Tree and Heisler intestinal contents and soil samples were measured by AODC and MPN viable counting.

<sup>b</sup> TOC, total organic carbon. Data are from analysis by the Soil Testing Laboratory, Michigan State University, unless otherwise noted.

<sup>c</sup> Values are  $\log \cdot g^{-1}$  of sample (wet weight). Average standard deviation is 74% of mean.

<sup>d</sup> Values are  $\log \cdot g^{-1}$  of sample (wet weight). Aer., aerobic; Ana., anaerobic. 95% CI =  $\pm 0.52$ .

<sup>e</sup> J. Ogden, Dalhousie University.

<sup>f</sup> ND, not determined.

<sup>g</sup> —, none detected in duplicate analyses.

media were prepared according to the procedures of the Virginia Polytechnic Institute anaerobe manual (8) with the following modifications: O<sub>2</sub>-free CO<sub>2</sub> atmosphere, 100%; NaHCO<sub>3</sub> replaced with 0.4% Na<sub>2</sub>CO<sub>3</sub>. *Clostridium* egg yolk agar and *Bacteroides* bile esculin agar were prepared following the formulations detailed in the *Manual of Clinical Microbiology* (6).

**Enumeration.** The number of organisms present in each sample was determined by both acridine orange direct counting (AODC) and most-probable-number (MPN) viable counting. Aliquots of the initial sample suspensions were homogenized anaerobically with a sterile glass tissue grinder and serially diluted into serum bottles containing PRAS dilution fluid (8) for viable counts or into tubes containing Tris-EDTA buffer (pH 8.0) for direct counts. Direct counts were obtained in accordance with the procedures of Schmidt and Paul (14) with black 25-mm-diameter, 0.22-mm-pore-size polycarbonate filters (Millipore, Bedford, Mass.). MPN estimates of abundance were determined for separate five-tube inoculations containing either PRAS BHI or BHI medium. These media were inoculated with 0.1 ml of each serial dilution and incubated at 25°C until no changes in turbidity were observed (1). Additionally, duplicate EMB spread

plates were inoculated with 0.1 ml from each serial dilution to determine the abundance of coliform bacteria.

**Enrichment.** Aliquots of each frozen sample were aseptically transferred into tubes containing ca. 7.0 ml of PRAS dilution fluid under an O<sub>2</sub>-free CO<sub>2</sub> atmosphere. Samples were allowed to thaw at room temperature under strict anaerobic conditions. Once thawed, 1.0 ml of inoculum was transferred anaerobically into three 100-ml serum bottles, each one containing 1/100-strength PRAS SE (1/100 PRAS SE), 1/10 PRAS BHI, or 1/10 aerobic BHI media, by using a large-bore pipette. Anaerobic SE enrichments were incubated at 37°C, while BHI enrichments were prepared in duplicate and incubated at 25 and 37°C, respectively. Enrichments were observed daily for visible evidence of growth. When turbidity was observed, approximately 1.0 ml of inoculum was transferred into fresh 1/10 PRAS SE, full-strength PRAS BHI, and full-strength aerobic BHI media as appropriate. Incubation conditions were repeated as above. The two-step enrichment was later omitted and replaced by a single enrichment in medium containing either 1/10 SE and 1/10 BHI or 1/10 SE and 1/10 chopped meat-glucose.

**Isolation and identification.** Following incubation, all enrichments were streaked for isolation onto general and selective media. Aerobic enrichments were plated onto nonselective BHI agar and enteric-selective MacConkey and BG agars. Single colonies, representing every unique colony type, were chosen from each plate based on colonial morphology or reactions to selective media. Anaerobic enrichments were streaked onto PRAS BHI and SE media for the isolation of general anaerobic bacteria and onto *Clostridium* egg yolk agar and *Bacteroides* bile esculin agar and for the selective isolation of *Clostridium* spp. and *Bacteroides* spp., respectively. All anaerobic isolations were performed in an anaerobic glove box under a 90% N<sub>2</sub>:10% H<sub>2</sub> atmosphere. Presumptive obligately anaerobic isolates were confirmed by comparing growth on aerobic BHI agar and PRAS BHI agar plates. All isolates were purified by at least two transfers on either BHI or PRAS BHI agar. Purified isolates were stored as stock cultures in 10% glycerol-BHI medium or in PRAS BHI agar stabs at -70°C. Isolates were characterized by Gram stain, motility, cellular morphology, and oxidase and catalase reactions (aerobes only).

Aerobic and anaerobic isolates were primarily identified by cellular fatty acid-methyl ester (FAME) analyses. Identities of aerobic isolates were confirmed initially with either Enterotube or Oxi-Ferm tubes (Roche Diagnostic Systems, Bellville, N.J.) and later with API-20E or Rapid NFT test strips (Analytab Products, Plainview, N.J.). Working cultures for FAME analyses were maintained on trypticase soy agar slants (BBL, Cockeysville, Md.) or anaerobic BHI stabs. Cell pellets were harvested, saponified, and methylated, and FAME profiles were analyzed using commercially available protocols and the gas chromatograph-software system, Microbial Identification System (MIDI) (Microbial ID Incorp., Newark, Del.).

**16S rDNA sequencing.** Comparative 16S ribosomal DNA (rDNA) sequencing was used to better identify some obligately anaerobic isolates for which there was no match in the MIDI database and to confirm the identification of selected aerobic isolates identified as members of the *Enterobacteriaceae* by FAME and/or biochemical analysis. Partial sequences from the type strains of *Enterobacter cloacae* and *Rahnella aquatilis* (ATCC 13047 and 33071, respectively) were also generated for comparison to isolates from the mastodon remains. All mastodon isolates used for rDNA sequencing were subcultured from frozen, glycerol stocks. Aerobes were grown on BHI agar and anaerobes were grown on PRAS BHI agar as described above. Near-complete (ca. 1,500 bp) small subunit rRNA genes were PCR amplified with the eubacterial-specific primers and under the conditions previously described (16). Excess amplification primers were removed with commercially available columns (Wizard PCR Preps; Promega, Madison, Wis.) prior to sequencing as per the manufacturer's instructions. Partial sequences of PCR products were obtained by automated, fluorescent *Taq* cycle sequencing with a 373A DNA Sequencer (Applied Biosystems, Foster City, Calif.) and a reverse-sequencing primer (5'-CGCGGCTGCTGGCAC-3') targeted to a conserved region of the 16S rRNA gene (positions 515 to 529 in *Escherichia coli* numbering). Approximately 400 unambiguous nucleotide positions near the 5' end of the gene were used for comparison of aerobic isolates, and approximately 450 unambiguous positions were used for comparison of anaerobic isolates (GenBank accession no. U65705 to U65721). Sequences from nearest relatives were identified and were obtained from the Ribosome Database Project (RDP) by using the SIMILARITY\_RANK and SUBALIGNMENT programs, respectively (11). Sequences were then manually brought into alignment based upon both primary and secondary structures by using the GDE editor obtained from the RDP. Percent similarities between aligned sequences were determined by using the AE2 program also obtained from the RDP (11).

## RESULTS

Differences between AODC and MPN population density estimates for each sample ranged over several orders of magnitude (Table 1). These differences are not surprising, and most investigators agree that AODC analyses overestimate viable population densities due to staining of nonculturable cells and interferences from soil organic matter. Average Burning Tree AODC population densities in putative intestinal contents and bone-associated samples were 9.24 and 8.87 log bacteria/g of sample, respectively. This is approximately the same order of magnitude, and statistical analysis revealed no significant differences between these samples. The average AODC values for background sediments were significantly different from intestinal mass samples at the 90% level of significance. No statistical difference was found between AODC values for bone-associated and sediment samples. Aerobic MPN analyses showed greater distinctions among Burning Tree intestinal contents, bone-associated samples, and background sediment samples with averages of 7.44, 4.21, and 4.08 log bacteria/g of sample, respectively. The average aerobic MPN value for the intestinal mass was statistically significantly

different from that for the bone-associated samples at the 99% level and from that for the sediment samples at the 95% level. Differences among the anaerobic MPN data resemble the differences observed among the AODC analyses. The differences among the average anaerobic MPN values for intestinal contents, bone-associated samples, and background sediments (5.00, 3.77, and 3.36 log bacteria/g of sample respectively) were statistically significant only for intestinal mass and sediment samples (90% level of significance). This illustrates the unique habitat provided to bacteria by the intestinal contents.

The average AODC values for samples from the Heisler site were 8.06 and 7.72 log bacteria/g of sample for clastic anchors and background sediments, respectively, and did not reveal a significant difference based on the origin of the samples. Statistically significant differences were observed between the average MPN values for aerobic and anaerobic bacteria. Average MPN values under aerobic conditions were 6.55 and 4.72 log bacteria/g of sample, and average MPN values for anaerobic incubations were 4.37 and 3.03 log bacteria/g of sample for clastic anchors and sediments, respectively. In both cases, average differences were significant at the 90% level. Although statistically significant, the differences in bacterial populations at the Heisler site were not as great as those observed for samples from the Burning Tree site and indicate that clastic anchor and sediment habitats are more similar than comparable intestinal masses found at the Burning Tree site.

Organisms isolated from mastodon remains and background sediments represent numerous species of bacteria (Table 2). For convenience, isolates are grouped according to the major taxonomic sections in *Bergey's Manual of Systematic Bacteriology* (9). In total, 295 isolates were analyzed. The MIDI database produced excellent matches (matching index, >0.6) for 96 isolates (33%) and matches with a strong likelihood (matching index, 0.5 to 0.6) for 28 isolates (9%). Matches signifying closely related species (matching index, 0.3 to 0.5) were determined for 43 isolates (15%). FAME profiles of 128 isolates (43%) could not be matched to any profile in the MIDI library (matching index, <0.3). Identity of an isolate was considered acceptable when the FAME profile matched one of those in the MIDI database at a matching index of >0.5. In all, 189 strains were identified; 124 were satisfactorily identified by FAME analysis with another 65 being added to the list after confirmation on the basis of biochemical tests and 16S rDNA sequencing. The majority of these isolates were facultatively anaerobic gram-negative rods (41%) and aerobic gram-negative rods (39%). Irregular, nonsporing, gram-positive rods, endospore-forming gram-positive rods, gram-positive cocci, and nonphotosynthetic, nonfruiting gliding bacteria accounted for 5, 5, 4, and 7% of the remainder, respectively. No coliform bacteria were directly cultivated from unenriched suspensions of Burning Tree intestinal material or Heisler clastic anchor samples on EMB agar.

FAME profiles from anaerobes were particularly difficult to match to those in the MIDI database. MIDI identified 29, 5, and 4 anaerobes as members of the genera *Bacteroides*, *Fusobacterium*, and *Clostridium*, respectively. Representatives of these isolates with the highest matching indices were subsequently analyzed by gas chromatography for volatile fatty acid fermentation products (data not shown). Fermentation products were consistent with the identification of *Clostridium* isolates but were inconclusive or contradictory for other isolates. Subsequently, nine of these obligate anaerobes were selected for comparative 16S rDNA sequencing. All were identified as members of the genus *Clostridium* by partial 16S rDNA sequence (Table 3) and were most closely related to clostridial species previously isolated from soils.

TABLE 2. Representative species of bacteria isolated from intestinal mass, bone-associated, and sediment samples from the Burning Tree and Heisler mastodon sites

Bacterium <sup>b</sup>	Presence of bacterium in <sup>a</sup> :																	
	Pooled sample						Individual sample											
	Burning Tree			Heisler			Burning Tree						Heisler					
	IM	B	S	IM	S	IM		B		S		CA		S				
						1 <sup>c</sup>	2	6	7	4	8	10	12	13	14	17	18	20
Facultatively anaerobic gram-negative rod																		
<i>Enterobacter agglomerans</i>	•	•				•												
<i>Enterobacter cancerogenus</i>		•	•	•					•	•		•			•			
<i>Enterobacter cloacae</i>	•	•			•	•	•	•	•	•								•
<i>Hafnia alvei</i>	•							•										
<i>Klebsiella planticola</i>	•			•		•										•		
<i>Serratia liquefaciens</i>		•								•								
<i>Serratia plymuthica</i>	•	•							•	•								
<i>Yersinia enterocolitica</i>	•	•				•				•								
Gram-negative aerobic rod																		
<i>Acinetobacter baumannii</i>	•	•				•				•								
<i>Alcaligenes xyloxydans</i>	•						•	•										
<i>Bordetella bronchiseptica</i>	•					•												
<i>Comamonas acidovorans</i>	•	•				•	•			•								
<i>Comamonas testosteroni</i>				•													•	
<i>Pseudomonas aeruginosa</i>			•											•				
<i>Pseudomonas aureofaciens</i>	•	•		•		•			•	•							•	
<i>Pseudomonas chlororaphis</i>	•						•		•									
<i>Pseudomonas coronafaciens</i>	•					•												
<i>Pseudomonas facilis</i>		•								•								
<i>Pseudomonas marginales</i>		•							•	•								
<i>Pseudomonas multivorans</i>		•								•								
<i>Pseudomonas putida</i>	•					•	•	•	•									
<i>Pseudomonas syringae</i>	•					•												
Nonsporing, gram-positive rod																		
<i>Arthrobacter aureescens</i>	•						•											
<i>Arthrobacter crystallipolites</i>		•								•								
<i>Aureobacterium barkeri</i>		•								•								
<i>Clavibacter michiganensis</i>		•								•								
<i>Corynebacterium aquaticum</i>	•						•											
<i>Curtobacterium flaccumfaciens</i>	•						•											
Endospore-forming gram-positive rod																		
<i>Bacillus coagulans</i>	•						•											
<i>Bacillus subtilis</i>				•													•	
<i>Clostridium bif fermentans</i>				•														
<i>Clostridium magenotii</i>		•	•							•		•		•		•		
<i>Clostridium subterminale</i>			•									•		•				
<i>Clostridium xylanolyticum</i>					•													•
Gram-positive coccus																		
<i>Micrococcus luteus</i>	•		•				•											•
<i>Micrococcus lylae</i>	•						•											
<i>Micrococcus roseus</i>			•															•
Nonphotosynthetic, nonfruiting gliding bacterium																		
<i>Sphingobacterium multivorum</i>		•								•								

<sup>a</sup> Isolates from individual samples were combined to form a composite based upon sample origin. IM, intestinal mass; B, bone-associated sample; S, sediment; CA, clastic anchor. Isolates are not reported where the MIDI similarity indices fall below the level of acceptable identification (<0.5).

<sup>b</sup> Initial identifications of isolates were determined by FAME analyses. Identifications of facultatively anaerobic gram-negative rods were confirmed by API-20E profiles.

<sup>c</sup> Sample number.

Eight isolates identified as members of the *Enterobacteriaceae* by API-20E and FAME profiles (matching index range, 0.624 to 0.135) were also sequenced to confirm these identifications. Of five isolates identified as *Yersinia enterocolitica*,

three had identical sequences over the region analyzed and were most closely related to *R. aquatilis* (99.8% sequence similarity), a close relative of the genus *Yersinia* and a member of the yersinia subgroup of the RDP. A fourth isolate, though

TABLE 3. 16S rDNA analysis of mastodon isolates

Sample no.	Origin <sup>a</sup>	Isolate	FAME identification	MIDI index	Biochemical identification	Closest relative (16S rRNA)	% 16S rRNA similarity
<i>Enterobacteriaceae</i> isolates							
Burning Tree							
1	IM	101.03.37	<i>Enterobacter cloacae</i>	0.135	<i>Enterobacter cloacae</i> <sup>b</sup>	<i>E. coli</i>	95.4
		101.05.25	<i>Y. enterocolitica</i>	0.512	<i>Pseudomonas</i> sp. <sup>b</sup>	<i>R. aquatilis</i>	99.8
		101.07.25	<i>Y. enterocolitica</i>	0.584	<i>Pseudomonas</i> sp. <sup>b</sup>	<i>R. aquatilis</i>	99.8
		102.05.25	<i>Y. enterocolitica</i>	0.624	<i>Pseudomonas</i> sp. <sup>b</sup>	<i>R. aquatilis</i>	99.8
6		E6.25.18	<i>Yersinia</i> sp.	0.297	ND <sup>c</sup>	<i>R. aquatilis</i>	99.3
4	B	401.04.37	<i>Enterobacter cloacae</i>	0.477	<i>Aeromonas salmonicida</i> <sup>d</sup>	<i>E. coli</i>	95.6
	B	402.03.25	<i>Enterobacter cloacae</i>	0.761	<i>Enterobacter cloacae</i> <sup>b</sup>	<i>C. freundii</i>	98.0
Heisler							
18	CA	18.37.BG.02	<i>Y. enterocolitica</i>	0.351	<i>Enterobacter</i> sp. <sup>e</sup>	<i>H. alvei</i>	97.8
Obligately anaerobic isolates							
Burning Tree							
8	B	8.37.71	<i>Bacteroides</i> strain D19	0.069	ND	<i>C. mangenotii</i>	98.5
12	Sed	12-13	<i>Bacteroides</i> strain D19	0.294	ND	<i>C. subterminale</i>	92.4
		12-6	<i>Fusobacterium necrophorum</i>	0.001	ND	<i>C. subterminale</i>	91.7
		12-12	<i>Bacteroides</i> strain D19	0.168	ND	<i>C. mangenotii</i>	98.5
		14-1	<i>Fusobacterium necrophorum</i>	0.003	ND	<i>C. subterminale</i>	92.6
14	Sed	14-2	<i>Bacteroides</i> strain D19	0.080	ND	<i>C. subterminale</i>	91.4
		14-19	<i>Bacteroides</i> strain D19	0.138	ND	<i>C. mangenotii</i>	98.1
Heisler							
17	CA	17-3	<i>Bacteroides</i> strain D19	0.008	ND	<i>C. bif fermentans</i>	98.9
22	Sed	22-6	<i>F. perfoetens</i>	0.032	ND	<i>C. xylanolyticum</i>	98.3

<sup>a</sup> IM, intestinal mass; B, bone-associated sample Sed, background sediment; CA, clastic anchor.

<sup>b</sup> API-20E test strip.

<sup>c</sup> ND, not determined.

<sup>d</sup> Rapid NFT test strip.

<sup>e</sup> Enterotube.

more distantly related to the others, was also most closely related to *R. aquatilis* (99.3% sequence similarity). A fifth isolate was most closely related to *Hafnia alvei* (97.8% sequence similarity). Three isolates identified as *Enterobacter cloacae* by MIDI were found to be most closely related to either *E. coli* or *Citrobacter freundii* (95.4 to 98.0% sequence similarity) (Table 3). The type strain of *Enterobacter cloacae*, on the other hand, was more distantly related (94.4 to 97.7% sequence similarity) to these isolates. Table 3 summarizes the results of the various identification methods for these isolates.

Intestinal and bone-associated samples from Burning Tree account for the richest taxonomic diversity of isolates (Tables 2 and 4). Numerically, pooled samples of intestinal contents and bone-associated and sediment isolates were represented by 21, 17, and 6 taxa, respectively, while pooled samples from Heisler accounted for 6 species from intestinal samples and 2 species from sediments. A comparison of the richness of isolates representing major groups of bacteria showed that facultatively anaerobic gram-negative rods were a major component of samples associated with the Burning Tree mastodon remains (Table 1). Of the enteric taxa found in the Burning Tree intestinal masses and bone samples, four taxa were present in both. Of the other isolates, *H. alvei* and *Klebsiella planticola* were unique to the intestinal masses while *Enterobacter cancerogenus* and *Serratia liquefaciens* were only found in bone samples. Gram-negative aerobic rods were also more diverse in Burning Tree intestinal and bone samples than in background sediments; however, more individual taxa were found in this group than in the enteric group described above. These two groups showed more diverse assemblages of taxa in mastodon

TABLE 4. Number of bacterial taxa cultured from Burning Tree and Heisler mastodon samples representing major taxonomic groups

Group	No. (%) of bacterial taxa in sample from:				
	Burning Tree			Heisler	
	Intestine	Bone	Sediment	Clastic Anchor	Sediment
Facultatively anaerobic gram-negative rods	6 (29)	6 (35)	1 (16)	2 (33)	1 (50)
Gram-negative aerobic rods	9 (42)	6 (35)	1 (16)	2 (33)	— <sup>a</sup>
Nonsporing, gram-positive rods	3 (14)	3 (18)	—	—	—
Endospore-forming gram-positive rods	1 (5)	1 (6)	2 (34)	2 (33)	1 (50)
Gram-positive cocci	2 (10)	—	2 (34)	—	—
Nonphotosynthetic, nonfruiting gliding bacteria	—	1 (6)	—	—	—
Total	21 (100)	17 (100)	6 (100)	6 (100)	2 (100)

<sup>a</sup> —, none detected.

remains than in the background sediment. Many of the Burning Tree bone-associated strains were pseudomonads and most likely represent opportunistic invaders from the environment. Gram-positive organisms predominated in sediment samples from both sites. In particular, endospore-forming rods, represented by *Bacillus* spp. and *Clostridium* spp., accounted for 34% of the Burning Tree sediment taxa and 50% of taxa from Heisler sediments. Samples from Heisler, overall, produced few isolates, and clear distinctions between clastic anchors and background sediments are not as evident in our microbiological data.

## DISCUSSION

The number and composition of bacterial species isolated from intestinal materials collected at the Burning Tree site are consistent with the macrobotanical analysis in implicating the intestinal origin of this material. Of all the isolates cultivated, *Enterobacteriaceae* predominate, indicating that enrichment and isolation procedures successfully yielded a collection of organisms with the greatest probability of being representative of intestinal microbiota. The high proportion of enteric organisms in the intestinal masses could either be derived from the animal's gut microbiota or represent postmortem invaders enriched on the carcass carbon. While we are unable to conclusively distinguish between these two inoculum sources, the low proportion of these organisms found in adjacent sediments suggest that the current, enriched population derives from the time of the animal's death.

Comparative 16S rDNA sequencing was used to test the reliability of the MIDI identification of eight isolates as members of the *Enterobacteriaceae*. Ribosomal sequence confirmed MIDI classification of these isolates as members of the *Enterobacteriaceae*, although it also suggested different species identifications. This is not surprising in light of the low matching indices of some of the isolates by conventional tests (Table 3). Four of the five identified as members of the genus *Yersinia* by MIDI were identified as *R. aquatilis* (99.3 to 99.8% sequence similarity), the nearest relative to the *Yersinia* (based upon 16S rRNA phylogeny) (11). Although some isolates of *R. aquatilis* have been isolated from rhizosphere and fresh water, the mastodon isolate sequences were nearly identical to more distantly related strains isolated from human bronchial aspirates (12). Our analysis of the type strain of *R. aquatilis* showed its rDNA sequence to be more similar to those from the environmental isolates. The fifth isolate, identified as *Y. enterocolitica* by MIDI, was most closely related to *H. alvei*, a common inhabitant of the gut. The three isolates identified as *Enterobacter cloacae* by MIDI were confirmed as enterics by rDNA sequence and were most closely related to either *E. coli* or *C. freundii* (Table 3), both also common intestinal flora. The occurrence of endospore-forming, obligately anaerobic bacteria in these samples supports the existence of both anaerobic and harsh survival conditions during the history of these sediments. Since the richness of bacteria is low in background sediment samples, we reason that survival in this habitat is not frequent apart from the protective habitat provided by the intestinal mass and residual tissues. The low diversity of endospore-forming organisms within the intestinal mass and bone-associated samples indicates that transport of bacteria from the background pond sediment into the intestinal remains was not extensive. The high diversity of gram-negative aerobic rods in the intestinal samples in comparison to background sediments also indicates that no recent extensive transport between these samples has occurred.

The lower numbers and diversity of bacteria in the Heisler

clastic anchors may reflect the disturbance to this assemblage posed by introduction of sand and gravel, derived from sub-aerial exposures beyond the pond margin (2, 3), into the intestinal lumen and restriction of intestinal contents to a relatively thin veneer lining the former interior of the intestinal wall. This veneer was especially tenuous in Heisler sample 18, in contrast to sample 17 (Table 1). By identifying intestinal materials at these sites, we force consideration of the nature of their association with skeletal remains. The clastic anchor hypothesis covers this issue for the Heisler site but the intestinal mass at the Burning Tree site seems inappropriate for such use. A possible interpretation of the Burning Tree occurrence was suggested by recent butchering and meat-caching experiments (3). A piece of intestine of a length somewhat greater than local water depth is capable of acting as a marker buoy, anchored to the pond bottom by intestinal contents, while its opposite end floats, marking the position of a submerged cache of carcass parts to be retrieved later. In this interpretation, and for clastic anchors as well, the preservation of intestinal material is viewed as largely incidental to the human activities responsible for site formation. One issue that would be especially useful to resolve for evaluation of the meat-caching hypothesis is the amount of time that may have transpired between the death and dismemberment of the mastodon and the introduction of its carcass parts, and intestinal materials, into the pond environment.

A consequence of corroborating the hypothesis of intestinal origin is that paleobiologists interested in the diet of mastodons may now accept with greater confidence the macrobotanical assemblage described from these contexts as representative of mastodon diet for at least that portion of the year to which these samples provide access. Information on the diet of the victims of the late-Pleistocene megafaunal extinction is important for evaluating competing hypotheses for the cause of this major extinction event (7, 10).

In this study, numerous *Enterobacteriaceae* were cultivated from intestinal materials, indicating they are capable of long-term survival under certain environmental conditions. Dominant, obligately anaerobic, nonspore-forming, intestinal microbiota expected in the gut of large herbivores with cecal fermentation, however, were not cultivated and apparently are not as well adapted to long-term survival under these conditions. There is no way of determining how many generations the surviving bacterial strains may have experienced since the death of these mastodons. Once tissue-derived substrates were metabolized, we suspect that usable carbon sources were not available to bacterial survivors, and long-term survival mechanisms drastically reduced growth rates. Thus, any available sources of energy were likely used for cell maintenance and not growth.

This study provides microbiological evidence to support the hypothesis that the Burning Tree cylindrical mass is of intestinal origin. The origin of the Heisler samples, on the other hand, cannot be determined exclusively from the microbiological data. To date, few other studies have systematically examined the microbiology of remains associated with extinct mammal excavations. Future excavators should keep these findings in mind as proper sample collection and preservation are essential for rigorous microbiological analyses. Additional microbiological studies using samples with known carbon dates may provide key insights into the survival and evolution of bacteria in recent history.

## ACKNOWLEDGMENTS

We are grateful to Bradley T. Lepper and Paul E. Hooge, principal excavators of the Burning Tree site, for the invitation to study their

samples; S. G. Beld, and members of the Ann Arbor chapter of the Michigan Archaeological Society, for assisting Dan Fisher with the Heisler excavation; Sherman Byers and Lester and Jim Heisler, property owners of the Burning Tree and Heisler sites, respectively, for their cooperation; and T. W. Stafford, Jr., for processing bone samples for radiocarbon dating.

Financial support was provided through NSF grants DEB-9120006 and BNS-8521097.

#### REFERENCES

1. **Alexander, M.** 1982. Most probable number method for microbial populations, p. 815. *In* A. L. Page, R. H. Miller, and D. R. Keeny (ed.), *Methods of soil analysis*, no. 9 (part 2), 2nd ed. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, Wis.
2. **Fisher, D. C.** 1989. Meat caches and clastic anchors: the cryptic record of Paleoindian subsistence in the Great Lakes region. *Geologic Society of America Prog. Abstr.* vol. 21, p. A234.
3. **Fisher, D. C.** 1995. Experiments on subaqueous meat caching. *Curr. Res. Pleist.* **12**:77–80.
4. **Fisher, D. C., B. T. Lepper, and P. E. Hooge.** 1991. Taphonomic analysis of the Burning Tree mastodont. *Curr. Res. Pleist.* **8**:88–92.
5. **Fisher, D. C., B. T. Lepper, and P. E. Hooge.** 1994. Evidence for butchery of the Burning Tree mastodon, p. 43–55. *In* W. S. Dancey (ed.), *The first discovery of America: archaeological evidence of the early inhabitants of the Ohio area*. Ohio Archaeological Council, Columbus, Ohio.
6. **Forney, J. E., and J. M. Miller.** 1985. Quality control of culture media, p. 1054–1065. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of clinical microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.
7. **Guthrie, R. D.** 1984. Mosaics, allelochemicals, and nutrients: an ecological theory of the late Pleistocene megafaunal extinctions, p. 259. *In* P. S. Martin and R. G. Klein (ed.), *Quaternary extinctions: a prehistoric revolution*. University of Arizona Press, Tucson.
8. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. *Anaerobe laboratory manual*, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
9. **Krieg, N. R., and J. G. Holt.** 1984. *Bergey's manual of systematic bacteriology*. Williams & Wilkins, Baltimore, Md.
10. **Lepper, B. T., T. A. Frolking, D. C. Fisher, G. Goldstein, J. E. Sanger, D. A. Wymer, J. G. Ogden, and P. E. Hooge.** 1991. Intestinal contents of a late Pleistocene mastodont from midcontinental North America. *Quat. Res.* **36**: 1–6.
11. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. *Nucleic Acids Res.* **22**:3485–3487.
12. **Richard, C.** 1989. Nouvelles *Enterobacteriaceae* rencontrées en bactériologie médicale: *Moellerella wisconsensis*, *Koserella trabulsii*, *Leclercia adecarboxylata*, *Escherichia fergusonii*, *Enterobacter asburiae*, *Rahnella aquatilis*. *Ann. Biol. Clin.* **47**:231–236.
13. **Schaal, S., and W. Ziegler.** 1992. Messel. Clarendon Press, Oxford, United Kingdom.
14. **Schmidt, E. L., and E. A. Paul.** 1982. Microscopic methods for soil microorganisms, p. 803. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), *Methods of soil analysis*, no. 9 (part 2), 2nd ed. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, Wis.
15. **Snyder, G. S.** Personal communication.
16. **Zhou, J., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analysis of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus toluolyticus* sp. nov. *Int. J. Syst. Bacteriol.* **45**:500–506.