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Mycobacterium parascrofulaceum in Acidic Hot Springs in Yellowstone National Park▽

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Mycobacterium parascrofulaceum **was found in Norris Geyser Basin, Yellowstone National Park, in a system composed of two acidic (pH 3.0) springs with temperatures between 56°C at the source and 40°C at the confluence of both springs. Growth and survival assays at 56°C for 60 days were performed, confirming the origin of the strain.**

Environmental mycobacteria are normal inhabitants of a wide variety of habitats, including natural and municipal water and soil (5). This prevalence can be explained by their high innate resistance to chlorine and biocides in water distribution systems (3, 9) and by their capacities for biofilm formation. It is therefore possible for mycobacterial populations to persist in flowing water ecosystems in spite of their slow growth. Environmental mycobacteria also have extraordinary starvation survival (4, 8), persisting despite low nutrient levels in water. Furthermore, tolerance to extreme temperatures (7) results in the contamination of hot tap water, spas, and ice machines by environmental mycobacteria.

Even if mycobacteria are very common in water, soil, vegetation, and even acidic, brown-water swamps (2), in extreme environments, only one study has isolated several sequences belonging to mycobacteria in pore water extracted from rocks collected in Norris Geyser Basin, Yellowstone National Park (12), but no cultivable microbes have been recovered.

Our work was performed in two unnamed thermal springs and their respective runoffs in the One Hundred Spring Plain, Yellowstone National Park. This system of two springs (one green and one yellow) is very acidic (pH 3.0), with temperatures ranging in the green spring from 45.8°C (at the source) to 40.0°C (where it merges with the yellow spring). The yellow spring has temperatures of 56.1°C at the source and 40.0°C at the merging point.

A total of 18 water samples were collected in October 2005 (9) and June 2006 (11) in several locations along the temperature gradients of the two outflows from the thermal springs. Two-liter water samples were filtered on site with a portable water sampler (Cole-Parmer) through a 0.22-μm nylon membrane (Whatman). The filters were resuspended in 10 ml of the original spring water, stored in sterile plastic tubes, and kept refrigerated until arrival at the laboratory. Upon arrival, 1-ml aliquots from all samples were extracted with InstaGene matrix (Bio-Rad, Italy) and tested by PCR targeting of part of the 16S-23S spacer region. PCR primers sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′) and PCR conditions (denaturation for 5 min at 96°C and 38 cycles of 1 min of denaturation at 94°C, annealing at 59°C, and extension at 72°C) were described previously by Roth et al. (6). The remaining volume was concentrated by filtration through a 0.22-μm nylon membrane (Whatman). Concentrated sample was vortex eluted from the filter in 5 ml of sterile water, decontaminated by the Petroff method ($NaOH$-H_2SO_4) for 15 min at room temperature, and inoculated in liquid (Bactec Myco/F medium [Becton Dickinson] and BacT/Alert [bioMérieux, France]) and solid (Middlebrook 7H10; Becton Dickinson) cultures at 8°C, 22°C, 37°C, and 55°C for up to 42 days for the liquid and 60 days for the solid cultures. Negative cultures were concentrated and confirmed as negative by PCR. All positive cultures were confirmed by auramine-rhodamine fluorescence microscopy. Cultures found positive by microscopy were further isolated in Middlebrook medium, confirmed by PCR with the conditions described above, identified by GenoType mycobacterium CM and GenoType mycobacterium AS identification kits (Hain Lifescience BMGH, Germany), and sent to a commercial laboratory for sequencing. Sequences were compared with current database sequences (GenBank) by using the NCBI basic local alignment search tool (BLAST) to determine phylogenetic relatedness.

All the PCR amplifications from the original samples gave negative results, indicating the need for a culture-dependent method, probably due to the small amounts of mycobacteria present in the sample. *Mycobacterium* spp. were recovered from cultures at 22°C and 37°C. Nonmycobacterial contamination of cultures incubated at 55°C prevented the detection of mycobacteria at this temperature.

All laboratory procedures were subject to quality control procedures: positive and negative controls were used for all decontaminations and incubations. Duplicate samples were used for all assays.

Samples were positive in all the temperature gradients from the two springs, indicating a high adaptability of *Mycobacterium* spp. to temperature (Table 1). *M. scrofulaceum* was identified from the isolated strains by a GenoType mycobacterium CM kit. After sequence analysis, these identification results were not confirmed and were corrected to *M. parascrofulaceum*. With most of the conventional tests, differentiation of *M. scrofulaceum* from *M. parascrofulaceum* is problematic. The biochemical and cultural tests and high-pressure liquid chro-

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TABLE 1. Individual results for sampling sites

Sample no.	Spring identification or location	Sampling date	pH	Temperature (°C)	Organism(s) found or result
1	Yellow (near merging point)	October 2005	3.2	40.0	*M. parascrofulaceum*
2	Yellow (near merging point)	October 2005	3.0	39.5	*M. parascrofulaceum*
3	Green (near merging point)	October 2005	3.0	41.0	*M. parascrofulaceum*, *M. xenopi*
4	Green (near merging point)	October 2005	3.0	41.4	*M. parascrofulaceum*
5	Merging point	October 2005	3.0	38.9	*M. parascrofulaceum*
6	Merging point	October 2005	3.0	39.0	*M. parascrofulaceum*
7	Mixture	October 2005	3.1	37.8	Negative
8	Mixture	October 2005	3.0	39.4	*M. parascrofulaceum*
9	Yellow (near merging point)	June 2006	3.2	33.5	*M. parascrofulaceum*
10	Yellow (mid-spring)	June 2006	3.1	43.2	*M. parascrofulaceum*
11	Yellow (mid-spring)	June 2006	3.0	48.1	*M. parascrofulaceum*
12	Yellow (near source)	June 2006	3.0	56.1	*M. parascrofulaceum*
13	Green (near merging point)	June 2006	3.0	41.0	*M. parascrofulaceum*, *M. xenopi*
14	Green (source)	June 2006	3.0	45.8	*M. parascrofulaceum*, *M. xenopi*
15	Merging point	June 2006	3.3	40.0	*M. parascrofulaceum*
16	Merging point	June 2006	3.2	40.3	*M. parascrofulaceum*
17	Mixture	June 2006	3.2	39.3	*M. parascrofulaceum*
18	Mixture	June 2006	3.2	38.9	Contaminated

matography of mycolic acids are not discriminative. Both commercial DNA probes intended for the identification of *M. scrofulaceum* assign *M. parascrofulaceum* to that species (10). *M. parascrofulaceum* was first described by Turenne et al. (11), who isolated this species from clinical isolates. More recently, strains of *M. parascrofulaceum* were identified as the etiological agents responsible for several health problems in Italian and Spanish patients (10).

To confirm the temperature resistance of *M. parascrofulaceum* isolates, the strains were inoculated in 50 ml of Middlebrook 7H9 broth medium (Becton Dickinson) and incubated at 42°C and 55°C for 60 days. Samples were taken every 15 days and analyzed by auramine-rhodamine fluorescence microscopy to assess the presence of mycobacteria and with a Live/Dead bacterial viability kit (Molecular Probes) to assess bacterial viability. All samples were positive by both assays for the entire duration of the assay; therefore, we can conclude that the isolated mycobacteria can survive and persist at these temperatures.

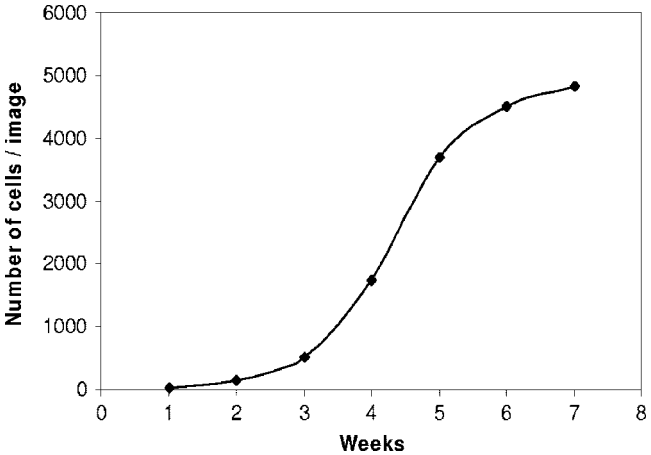

FIG. 1. Growth curve of *Mycobacterium parascrofulaceum* at 56°C.

Growth experiments were performed to confirm that isolated *M. parascrofulaceum* strains could grow at this temperature. A loopful of *M. parascrofulaceum* was inoculated in 5 ml of Middlebrook 7H9 medium and vortex homogenized. One hundred microliters of this solution was inoculated into eight Erlenmeyer flasks containing 50 ml of Middlebrook 7H9 and incubated at 56°C for 7 weeks. Every week, one Erlenmeyer flask was removed and the medium was centrifuged for 20 min at 5,000 × *g* in a Sigma 3-18K centrifuge (Sigma, Germany). The pellet was washed with distilled sterile water and resuspended in 100 μl of water. Samples were analyzed by auramine-rhodamine fluorescence microscopy with a Nikon Eclipse E400 microscope (magnification, ×400) (Nikon, Japan). Images were taken with a Nikon DXM 1200 camera (Nikon, Japan) with ACT-1 software (Nikon, Japan). Image analysis was carried out using Visilog 5 for Windows 95 from Noesis SA, Les Ulis, France, according to the method of de Carvalho et al. (1). Cell numbers increased (Fig. 1) from an initial concentration of 10 cells/image to 5,000 cells/image at week 7. This increase indicates that cultures are adapted to high temperatures, being able to grow at 56°C.

This is the first isolation of mycobacteria from extreme environments and the first isolation of *M. parascrofulaceum* isolates recovered from environmental sources.

One interesting feature of the Yellowstone isolates is their ability to survive in high temperatures compared with that of the isolates that were not able to grow at 42°C, as described in previous studies. This feature could indicate the possession of some temperature-related resistance mechanism that needs further investigation.

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