Enrichment for thermophilic bacteria from a not-so-unusual habitat (Soil near Swope Conference Center and a local playground) in Woods Hole, MA

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ABSTRACT

The original intention for this project was to isolate thermophilic actinomycetes from rather common habitats. They grow rapidly and can yield potentially useful industrial products. However, the organisms that predominated in the thermophilic environment were gram-positive rods, specifically of the genera Clostridia and Bacillus. These organisms were enriched selectively for chitin-degrading ability as well as growth at temperatures reaching 75°C, which would classify them as extreme thermophiles. The original enrichment growing at 55°C was analyzed at the molecular level. 16S rDNA sequences were amplified from prepared genomic DNA of the bacteria and sequenced. The sequences were shown to be related to Bacillus species and Clostridia by phylogenetic analysis. Also, flourescent *in situ* hybridization was performed that confirmed that and high (Bacillus) and low G+C gram-positive bacteria (e.g. Clostridia) were in the thermophilic samples.

INTRODUCTION

Thermophilic organisms have been studied extensively in recent years for many reasons. They can produce useful enzymes (e.g. Taq polymerase) as well as provide a clearer understanding of the origin of life, where the early biosphere would essentially select for thermophilic bacteria. This study was to try to analyze a rather simple environment and see if thermophiles existed in these environments. The conditions used were optimal for *Bacillus stearothermophilus*, but adding a molecular twist to this analysis would help determine if the assumption of this type of enrichment was indeed correct. *Bacillus* spp.and *Clostridia* spp. can form spores which survive high temperatures, but germinate usually with availability of a suitable substrate and a lowering of this temperature.

MATERIAL AND METHODS

Sample collection and enrichments. Using a Swiss army knife, I collected a 1-2 cm square soil sample from a plot near Swope Conference Center in Woods Hole, MA. This region was grassy and usually protected from direct sunlight by a tree lying near the sample. Another sample was taken from a local playground near some bushes. These samples were placed in petri dishes and incubated at 50°C for 16 hours. Approximately 0.5 g of soil was placed in a rich GYM (glucose-yeast extract-malt extract) medium in a parafilm-wrapped test tube and placed in a 55°C incubator. After initial growth in this rich media, these samples were subcultured into a minimal media, with acid-hydrolyzed chitin as the sole carbohydrate and nitrogen source. In additions, samples were subcultured into both types of media and placed in a 75°C incubator. For a final test of thermostability, the samples that grew at 75°C were placed into an incubator set for 85°C.

Microscopy. Samples were periodically analyzed by light and phase-contrast microscopy using a Zeiss microscope and the Metamorph imaging software on a PC.

Molecular and phylogenetic analysis. Genomic DNA was extracted from the samples growing at 55°C using a Mo Bio soil extraction kit. The 16S rDNA sequences from this genomic DNA was amplified by a polymerase chain reaction using eubacterial primers 8F and 1492R. The annealing temperature for the primers was 55°C. The PCR product had a hanging adenine residue (from actions of the Taq polymerase) which allowed efficient ligation into a TopoVector which had a corresponding hanging thymine residue. The ligation was catalyzed by a topoisomerase which had been covalently attached to the ends of the vector. This process was part of a new Topo ligation kit. The resulting vectors were transformed into *Escherichia coli* and plated onto LB-Amp plates. The DNA was amplified subsequently using M13 primers and product was electrophoresed on a 1.25% agarose gel to confirm product as well as correct size (500 bp of the ~1.5 kB 16S rDNA gene). Once these were confirmed, the products (6 in total) were sequenced using dye-terminator sequencing at the Forsyth Dental Center in Boston, MA. Two of these sequences were analyzed by the ARB program to create a

phylogenetic tree. The other four were analyzed by BLASTN searches for sequence homology.

FISH analysis. 250 µl samples from the 55°C cultures were fixed with 750 µl 4% paraformaldehyde. The samples were fixed for one hour at 4°C. Then, the samples were pelleted by centrifugation and washed once with ddH_2O. They were pelleted again by centrifugation and resuspended in 250 µl 95% ethanol. Approximately 20 µl of samples were placed onto a gelatin-coated slide and dried at 37°C. The slides were then soaked for 3 minutes each in the following ethanol series: 50% ethanol, 80% ethanol and 95% ethanol. The slides were air-dried and then a mixture of both hybridization buffer and either high G+C probe (CCYGATATCTGCGCA) or low G+C probe (TGTAGCCCARGTCATA) was pipetted onto the slides, upon which a coverslip was placed These probes have a flour attached which flouresces under the rhodamine (epi 3) filter. These slides were incubated overnight. The next day, the slides were washed in hybridization buffer equilibrated to 37°C, air-dried, and DAPI was added to the dried residue. After drying under a cover-slip, the slides were washed with water and a solution of ProLong™ (Molecular Probes, Oregon) was added to glue the coverslip to the slides and prevent bleaching of the probe. These slides were analyzed using epiflouresence microscopy [the rhodamine and DAPI filters (epi 3 and 1, respectively)].

RESULTS AND DISCUSSION

Using a rich medium for growth, I found many bacteria growing quite happily at 55°C. Analyzed under the microscope, these bacteria turned out to be a colossal arrangement or rods. Some rods (Figure 1) were assembled into long filament-like structures whereas others were single or double-rod structures. Both the Swope Soil and Playground soil had similar morphological types through microscopy. These original inoculums were subcultured into a chitin minimal media to determine if these thermophiles could use chitin as a sole carbon and nitrogen source. Growth was observed both at 55°C and 75°C. The samples at high temperatures were analyzed by microscopy and I saw a little less diversity in the rod structures. Most rods were single or double rods

(yet there were a few longer filament-type rod structures in the playground soil) (Figure 1) It seems that the ability to use chitin as a sole carbohydrate and nitrogen source selected somewhat for different morphological types, as well as the temperature. The selection for thermophilic Bacillus strains that can degrade chitin may be useful for isolation of different chitinases (2). When all these samples were placed at 85°C, everything lysed and ended as a mass at the bottom of the test or screw-cap tube.

The morphological types seen indicated that Bacillus and Clostridia were probably predominating in this thermophilic environment. I employed a variety of molecular techniques to determine if this was the case. By FISH analysis, I saw rod-shaped organisms in the samples which hybridized to the low G+C gram-positive probe. (Figure 2) The high G+C gram-positive probe was inconclusive, as no area had brighter intensity than background. This may be due to the inability of the probes to enter the gram-positive cell. The procedure detailed in ***Materials and Methods*** could be modified by adding lysozyme to the cells upon fixation or increasing the SDS concentration of the hybridization buffer. Analysis of the 16S rDNA sequences showed that I did indeed have *Bacillus* and *Clostridium* in the samples. All 3 samples for the Swope Soil showed homology to *Bacillus* spp. Two of the playground samples had homology to *Bacillus* and one had homology to a *Clostridium.* Figure 3 and 4 show phylogenetic trees as determined by sequence alignment and analysis using the ARB program for one sample each from the Swope Soil and Playground soil. The Swope Soil sample had as its nearest neighbor *Bacillus salexigens*, which is a moderately halophilic rod-shaped bacterium (4). The Playground soil sample was closest to a *Thermoanaerobacterium saccharolyticum*, which is a xylan-utilizing anaerobic thermophile (5)

This report, although simple in design, does show that thermophilic organisms are ubiquitous. Quite a variety of bacteria exist in both unusual and 'usual' environments. With the addition of molecular techniques, we might analyze some 'well-studied' environments further and really be able to determine what exists in the microbial world. If I had more time, I would have liked to determine the activity or presence of chitin-degrading ability by these thermophilic bacteria as well as the potential enzymes' thermostability. It is interesting that these organisms were growing at extremely thermophilic conditions. For example, a thermophilic actinomycetes could only grow up

to 68°C (3) Also to analyze whether the organisms are exclusively resistant to high temperatures or are inducing a type of overall stress response to their environment. One of the strains was closely homologous to a *Bacillus salexigens* which can grow under moderately high salt concentrations (4). This strain may be able to grow both at high temperatures and in highly saline environments. That can be studied by changing other environmental conditions and determining their effect on the microbial population, as well as the presence of certain heat-shock proteins or induction/repression of certain genes.

I would like to acknowledge Scott Dawson for fabulous help and information about FISH analysis, Tom Schmidt and Joel Klappenbach for help deciphering the sequence and producing a phylogenetic tree!!

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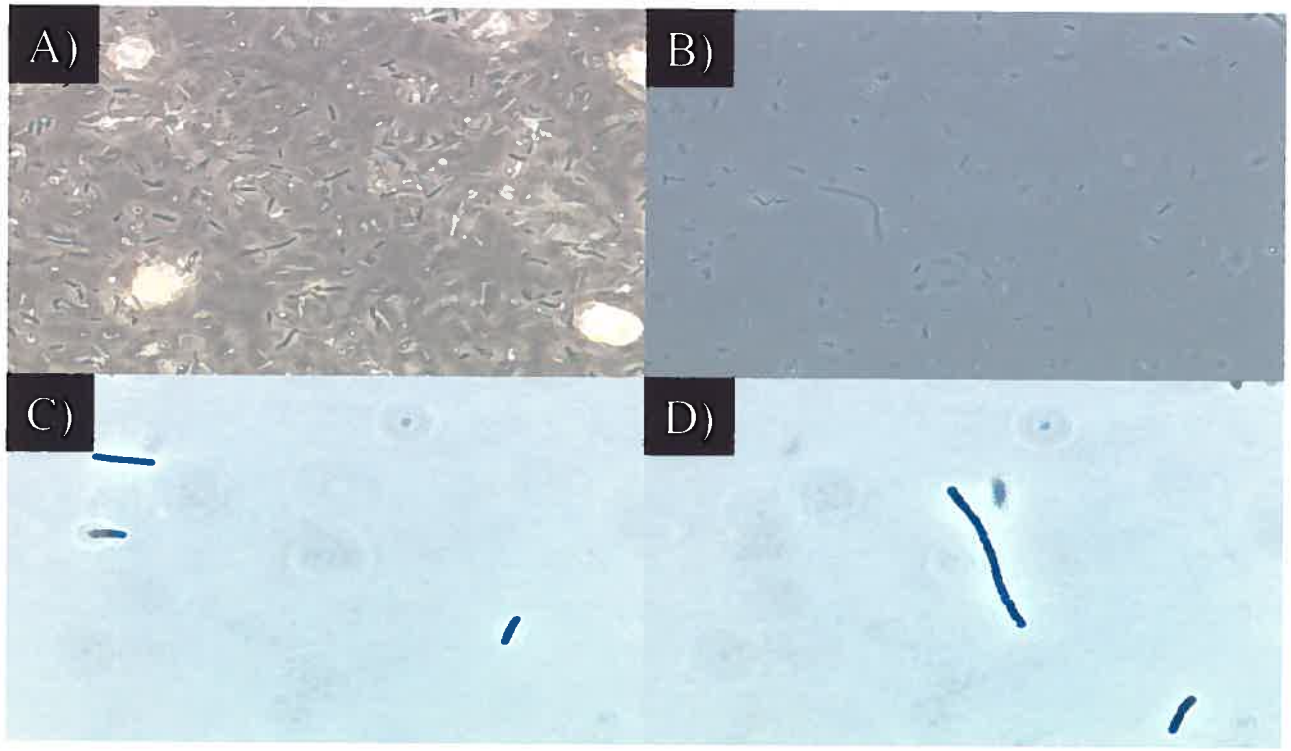
Thermophiles from Swope and Playground Soil.



A) Low magnification of Swope Soil at 55^0C
B) Low magnification of Playground Soil at 55^0C
C) High magnification of chitin-degrading bacteria at 75^0C
D) High magnification of chitin-degrading bacteria at 75^0C

Figure 1

FISH analysis of Swope Soil for Low G+C gram-positives at 55°C

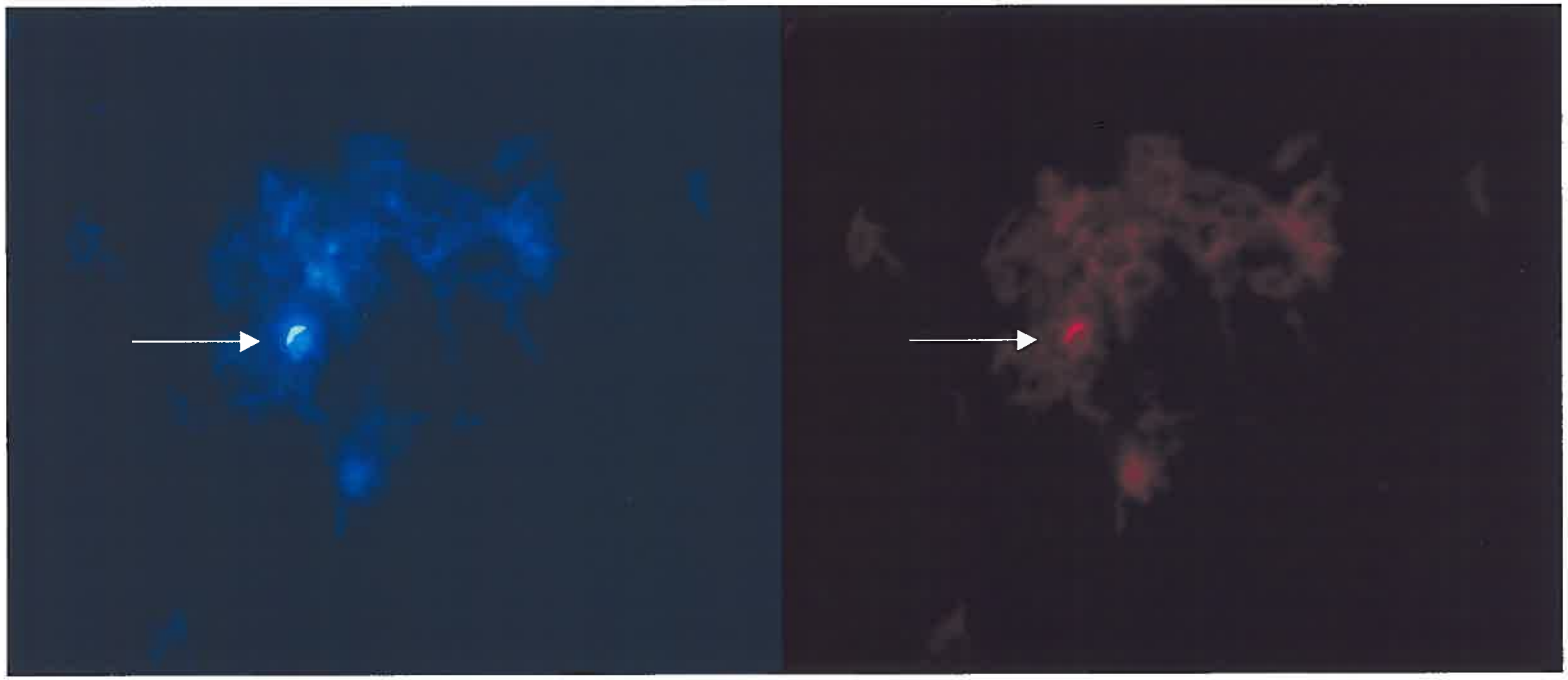


Figure 2

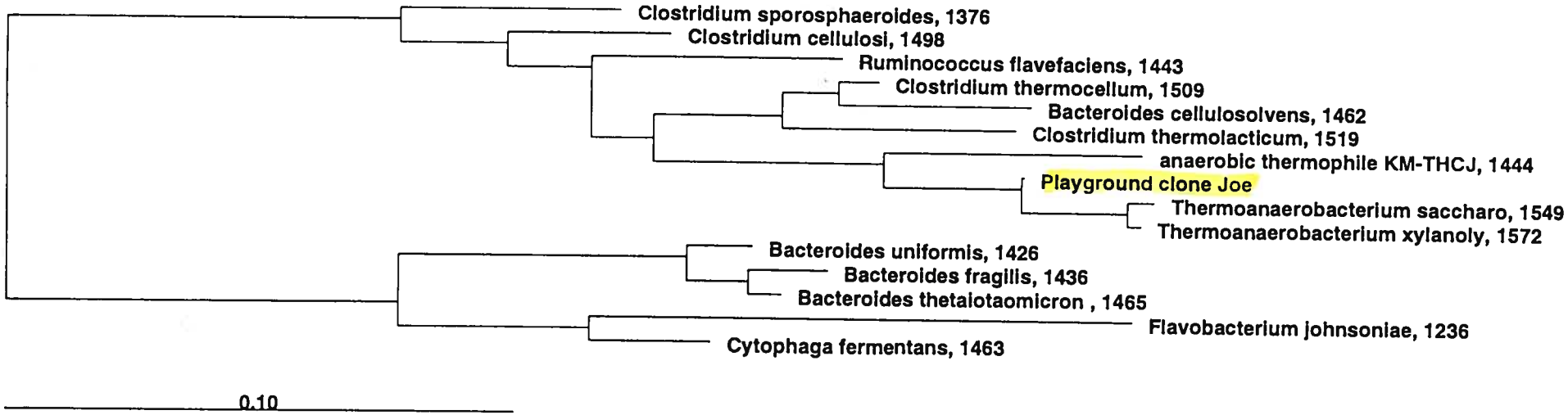

Clostridium sporosphaeroides, 1376
Clostridium cellulosi, 1498
Ruminococcus flavefaciens, 1443
Clostridium thermocellum, 1509
Bacteroides cellulosolvens, 1462
Clostridium thermolacticum, 1519
anaerobic thermophile KM-THCJ, 1444
Playground clone Joe
Thermoanaerobacterium saccharo, 1549
Thermoanaerobacterium xylanoly, 1572
Bacteroides uniformis, 1426
Bacteroides fragilis, 1436
Bacteroides thetaiotaomicron , 1465
Flavobacterium johnsoniae, 1236
Cytophaga fermentans, 1463
0.10

Figure 3

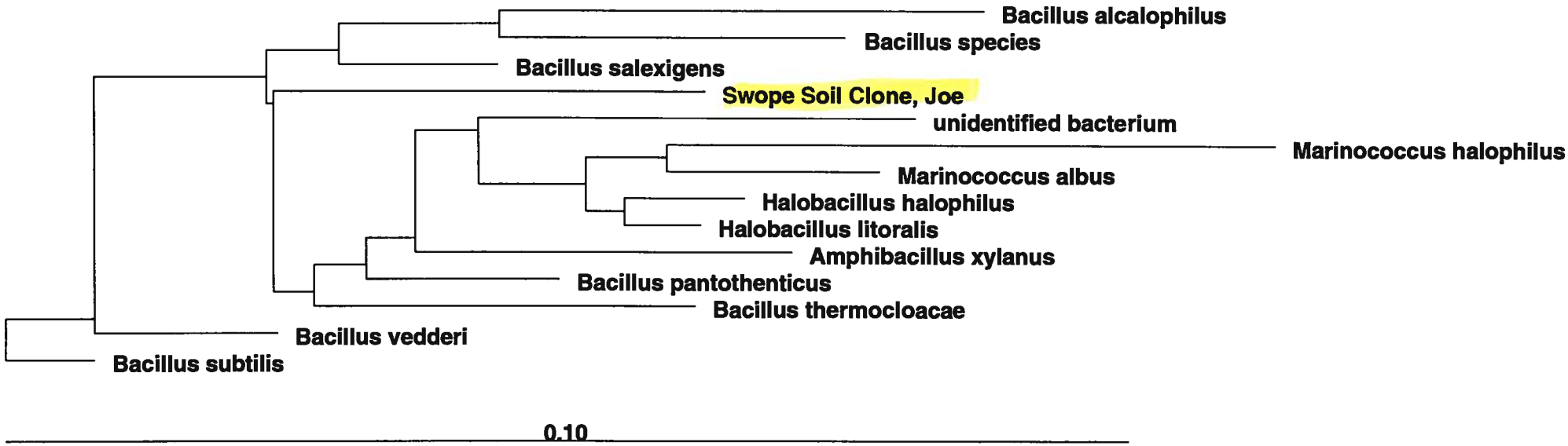

Bacillus alcalophilus
Bacillus species
Bacillus salexigens
Swope Soil Clone, Joe
unidentified bacterium
Marinococcus halophilus
Marinococcus albus
Halobacillus halophilus
Halobacillus litoralis
Amphibacillus xylanus
Bacillus pantothenticus
Bacillus thermocloacae
Bacillus vedderi
Bacillus subtilis
0.10

Figure 4