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EXPERIMENTAL ARTICLES

Characterization of Thermophilic Proteolytic Spore-Forming Bacteria from a Geothermal Site in Lithuania Based on 16S rDNA RFLP and ITS-PCR Analyses[1]

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Abstract—Forty-two strains of gram-positive, aerobic, heterotrophic, obligately thermophilic, spore-forming bacteria were isolated from a geothermal site near the Baltic Sea in Lithuania. All of the strains were able to hydrolyze collagen and/or casein. Since characteristics of proteolytic activity are correlated with taxonomic positions of bacteria, the strains were grouped on the basis of molecular biological analyses. On the basis of RFLP patterns of 16S rDNA and 16S–23S rDNA ITS-PCR analysis, the strains were subdivided into nine groups.

Key words: thermophilic, 16S rRNA gene, 16S–23S rDNA intergenic spacer region, RFLP analysis.

Thermophilic microorganisms are of great interest to enzymologists. They are the natural source of many thermostable enzymes, such as amylases, proteases, pullulanases, lipases, xylanases, glucose isomerases, glucosyltransferases and enzymes of DNA metabolism [1–4]. Thermophiles with multiple dioxygenases capable of degrading aromatic compounds have been isolated [5].

The purpose of our work was the detection and characterization of thermophilic microorganisms in a geothermal site near the Baltic Sea, with a focus on microorganisms producing thermostable proteases. In this communication, we report the isolation of thermophilic, aerobic, spore-forming bacteria capable of degrading collagen and/or casein. Because the bacteria were isolated from a natural environment and preliminary experiments indicated some differences between the bacterial strains, we expected our strains to belong to different taxonomic groups. Since different microorganisms have proteases with different characteristics, including different thermostability [6], we wished to avoid repeated examination of proteases in the same bacterial taxa. Therefore, we grouped and classified the strains on the basis of 16S rDNA restriction fragment length polymorphism (RFLP) analysis and 16S–23S rDNA internal transcription spacer PCR fingerprinting [7, 8].

[1]This article was submitted by the authors in English.

MATERIALS AND METHODS

Phenotypic characterization of strains. Size and morphology of vegetative cells and endospores were studied in fresh cultures (grown for 17–24 h at 60°C on nutrient agar) under an Olympus AX70 microscope. Gram staining was performed using the Merck kit; the Gram reaction was additionally checked by the express method with 3% KOH.

Minimum, optimum and maximum growth temperatures were established after cultivation at 40, 45, 50, 55, 60, 65, 70, 75, and 80°C. The medium used for this purpose was nutrient agar (Difco). NaCl requirements were tested in nutrient broth (Difco) at the following NaCl concentrations: 0, 5, 10, 15, and 20%. The strains isolated from the geothermal site were routinely stored at 4°C after growth on nutrient agar for 20–24 h at 60°C.

Casein digestion and catalase production were determined according to *Bergey's Manual of Systematic Bacteriology* [9]. Collagen digestion was determined in water agar (20 g/l) supplemented with 2% collagen.

DNA extraction. Bacterial genomic DNA was extracted from fresh cultures after cultivation on nutrient agar plates for 14 h at 60°C. The Genomic DNA Purification Kit (MBI Fermentas) was used for this purpose. Genomic DNA was extracted according to the manufacturer's instructions.

Amplification of 16S rDNA. Synthetic oligonucleotides were purchased from MBI Fermentas. Primers

0026-2617/02/7106-0712$27.00

Primers used for 16S rDNA and 16S–23S ITS amplification

Primer	Sequence (5'–3')	Nucleotides
27F	GAG AGT TTG ATC CTG GCT CAG	21
1495R	CTA CGG CTA CCT TGT TAC GA	20
S-D-Bact-1494-a-S-20	GTC GTA ACA AGG TAG CCG TA	20
L-D-Bact-0035-a-A-15	CAA GGC ATC CAC CGT	15

27F and 1495R (table) were universal primers for amplification of eubacterial 16S rDNA [10]. 16S rDNA was amplified in 50 μl of the reaction mixture, containing PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer, 1.25 U of recombinant TaqDNA polymerase, and 10 ng of bacterial genomic DNA. The reaction mixture was supplemented with 12% (v/v) DMSO. All reagents were provided by MBI Fermentas. Amplification was conducted in an Eppendorf thermal cycler under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles each consisting of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min, with a final extension step at 72°C for 7 min. Products of amplification were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide and visualized using a UV transilluminator.

RFLP analysis of 16S rDNA. Products from PCR primed by 27F/1495R were analyzed by single enzyme digestion according to the endonuclease manufacturer's instructions, with *Alu*I, *Hae*III, *Rsa*I, *Mse*I, and *Taq*I (MBI Fermentas). The RFLP profile of digested DNA was analyzed by electrophoresis through 5% polyacrylamide gel stained with ethidium bromide and visualized using a UV transilluminator.

Amplification of 16S–23S internal transcription spacers (ITS). Synthetic oligonucleotide primers were purchased from MBI Fermentas: forward S-D-Bact-1494-a-S-20 and reverse L-D-Bact-0035-a-A-15 (table) [11]. The reaction mixture and temperature profile were the same as for the amplification of 16S rDNA (see above). ITS amplification products were analyzed by electrophoresis in 1.5% agarose gel in TA buffer.

RESULTS

Phenotypic characteristics of strains. Vegetative cells of all strains were rods, in some strains occurring in chains. The size of vegetative cells was 1.34–0.84 by 0.13–0.09 μm. All the strains were gram-positive and formed endospores. Endospores were oval and central or subterminal, with little or no distension of the mother cell.

None of the 42 strains grew at 40°C; growth at 45°C was poor. Almost all isolates grew at 70°C; the exceptions were strains 1, 2, 5, 3.K, 27, 30, 35B, and 36B. Strains 17, 4.K, and 34 grew weakly at 70°C. The optimal temperature for all the strains was 55–65°C.

All strains grew with 5% NaCl in nutrient broth and none grew with 10, 15, or 20% NaCl.

All the strains were positive in the following tests: Gram reaction, endospore formation, catalase activity, and hydrolysis of collagen. Some of the strains (strains 1, 3, 4.K, 23, 35C, 3.K, 4, 14, 28, 6, 22, 25, 27, 31, N-3, 8, 32D, 33, 10, 1.K, and 36A) were also able to hydrolyze casein. Nineteen of the 42 strains exhibited high proteolytic activity. These strains were chosen for further study by RFLP analysis of 16S rDNA and by ITS-PCR analysis.

RFLP analysis of amplified 16S rDNA. The 19 strains exhibiting high caseinolytic and collagenolytic activity were differentiated on the basis of analysis of 16S rDNA. Collagenolytic activity was characteristic of all these strains, although caseinolytic activity could be demonstrated only in some strains (3, 35C, 4, 28, 6, 22, 25, 27, 31, N-3, 32D, 1.K, and 36A). RFLP analysis of 16S rDNA revealed similar *Mse*I, *Hae*III and *Rsa*I patterns for all of the 19 strains (data not shown). The *Alu*I RFLP patterns were indistinguishable for 17

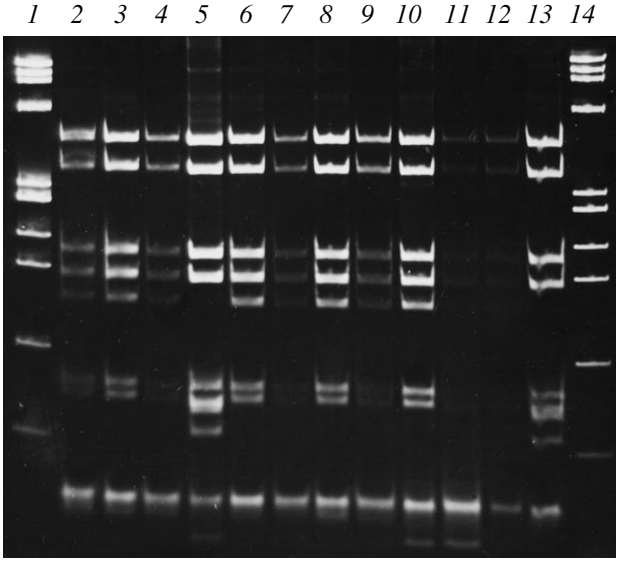

Fig. 1. *Alu*I RFLP profiles of 16S rDNA amplified in PCR primed by oligonucleotide pair 27F/1495R. Lanes *2–13*: strains 4, 17, 1.K, 22, 32A, 32B, 32C, 32D, 35A, 36A, 36B, and N-3. Lanes *1, 14*: marker ϕX174 DNA/*Bsu*RI(*Hae*III) digest, MBI Fermentas: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Data for strains 3, 6, 25, 27, 28, 31, and 35C are not shown.

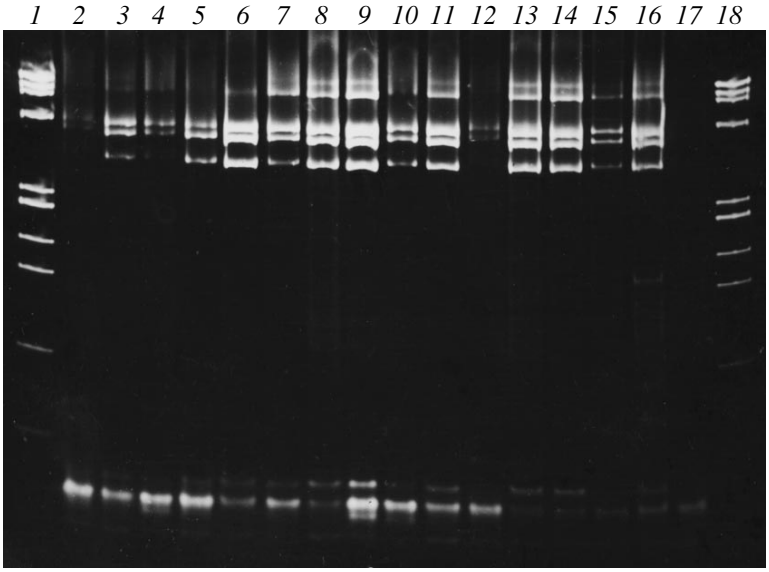

Fig. 2. *Taq*I RFLP profiles of 16S rDNA amplified in PCR primed by oligonucleotide pair 27F/1495R. Lanes *2–16*: strains 4, 17, 1.K, N-3, 22, 32A, 32B, 32C, 32D, 35A, 35C, 36A, 3, 27, and 28; lane *17*: control without DNA. Lanes *1, 18*: marker ϕX174 DNA/Bsu*RI*(*Hae*III) digest, MBI Fermentas: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Restriction profiles for strains 6, 25, 31, and 36B were identical to that of strain 28.

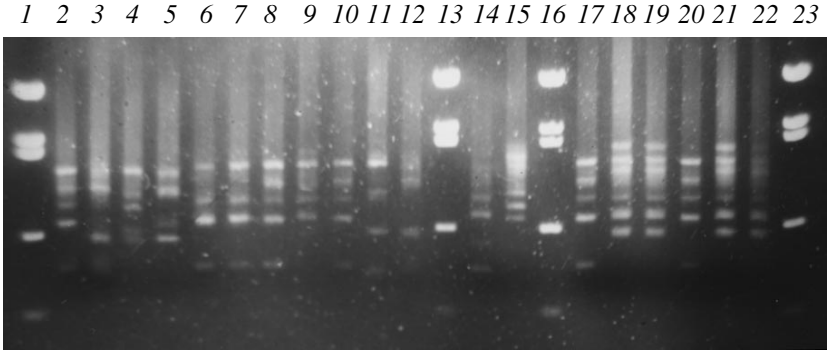

Fig. 3. 16S–23S rDNA ITS profiles after amplification in PCR primed by oligonucleotide pair S-D-Bact-1494-a-S-20/L-D-Bact-0035-a-A-15. Lanes *2–12*: strains 4, 17, 1.K, 32A, 32B, 32C, 32D, 35A, 35C, 36A, and 36B; lane *14*: strain N-3; lane *15*: strain 22; lanes *17–22*: strains 3, 6, 25, 27, 28, and 31. Lanes *1, 13, 16, 23*: marker pBR322 DNA/Mva*I*(Bst*NI*) digest, MBI Fermentas: 1857, 1058, 929, 383, 121.

strains, but they were different for two strains, 22 and N-3 (Fig. 1). In these latter patterns, the 160-bp fragment observed for all other strains tested was missing, and an 80-bp fragment was observed instead. *Taq*I RFLP analysis yielded five distinct patterns, distinguishing five separate groups among the 19 strains (Fig. 2). Restriction profiles for strains 22 and 1.K were unique. Strains 4 and 35C formed one cluster, and strains 32D and N-3 formed another. All the other strains were identical in their *Taq*I restriction patterns of 16S rDNA. Thus, RFLP analysis of 16S rDNA from 19 strains allowed us to distinguish of a total of six bacterial groups.

ITS-PCR profiles. After the amplification of the 16S–23S ITS region, no fragment larger than 900 bp was observed (Fig. 3). The profiles formed seven distinct patterns. Four distinct patterns were exhibited by the ITS regions of strains N-3, 22, 36A and 1.K; each of these patterns differed from patterns of all other strains. The remaining strains fell into three distinct groups with respect to their ITS patterns. Three strains (17, 32A, and 36B) represented the first of these patterns; four strains (6, 25, 28, and 31) exhibited the second pattern; and the remaining eight strains (4, 32B, 32C, 32D, 35A, 35C, 3, and 27) represented the third pattern.

Combined consideration of RFLP patterns of 16S rDNA and ITS-PCR patterns showed that the 19 bacterial strains examined by these molecular biological methods could be separated into nine groups.

DISCUSSION

In this communication, we report the isolation of diverse strains of heterotrophic, aerobic, thermophilic, spore-forming bacteria. Strains of bacteria that exhibit different restriction profiles of rDNA may represent different bacterial species [12]. Based on comparison of *Alu*I and *Taq*I RFLP patterns, our strains could be subdivided into six distinct groups. However, these differences might not reflect species-level distinctions in all cases. Thus, these our strains can be considered as representatives of six closely related species or of one or a few species, in each of which some variability exists in the rDNA sequences.

In comparative analyses of putative restriction profiles of 16S rDNA sequences of aerobic thermophilic spore-formers available from NCBI, we found that our strains possessed unique *Alu*I restriction patterns of rDNA. These patterns distinguished our strains from previously described strains whose sequences are available from NCBI. The *Hae*III, *Mse*I, *Rsa*I and *Taq*I RFLP patterns of 16S rDNA of our strains were identical to putative patterns of *Bacillus caldolyticus*, *B. vulcani*, and *Geobacillus subterraneus.* These findings indicate that our strains are related but distinct from these species.

ITS sequences are more variable than the adjacent genes and show significant variations at the genus or species level, and ITS-PCR profiles can be valuable for distinguishing closely related species [7, 8]. In prokaryotes, the ITS region is highly variable in length, as well as in nucleotide sequence [13]. Since this variability can be helpful for preliminary identification of bacteria, in our study ITS profiles were used with the aim of distinguishing our closely related strains of thermophilic bacteria. It is known that many gram-positive organisms have no tRNAs in the 16S–23S rRNA intergenic spacer region and thus have shorter ITS regions than gram-negative bacteria. Very rarely is the length of the spacer region above 500 bp in gram-positive species [13]. Our study demonstrated unusual ITS-PCR fingerprinting. While no ITS fragment larger than 900 bp was observed, in all patterns the major ITS fragment was 800 bp in size. This finding distinguishes our strains from other thermophilic aerobic spore-forming bacteria. Some strains of *Geobacillus* sp. exhibit ITS-PCR fragments larger than 500 bp. However, according to the data of Daffonchio *et al.* [7], the ITS-PCR pattern of *G. stearothermophilus* contains fragments of 230, 340, and 530 bp, and Manachini *et al.* [14] reported the presence of two major fragments of 625 and 450 bp in the *G. thermodenitrificans* ITS-PCR profile. Thus, no ITS profile of any of our strains was similar to the ITS profiles described for *G. stearothermophilus* and *G. thermodenitrificans.*

The results from 16S rDNA RFLP and ITS-PCR analyses suggest that all strains tested in the present study are closely related to one another. The proteolytic isolates could be subdivided into nine groups according to the results obtained. The clear molecular distinction between these groups revealed by the analysis of relatively highly conserved gene sequences (16S rDNA and the 16S–23S rRNA intergenic spacer region) is consistent with the possibility that they represent several distinct taxa. On this basis, we hypothesize that the groups differ in the characteristics of their extracellular proteases, since evolutionarily distinct taxa of microorganisms can possess different characteristics of their proteases [6]. Further investigations are needed to test this hypothesis, to accurately elucidate the degree of relatedness between our strains, and to elucidate their phylogenetic position in relation to that of other aerobic thermophilic spore-formers.

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