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Specific Detection of the Gene for the Extracellular Neutral Protease of *Bacillus cereus* by PCR and Blot Hybridization

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A pair of primers and a gene probe for the amplification and detection of the *Bacillus cereus* neutral protease gene (NPRC) were developed. Specificity for the *npr* genes of the *B. cereus* group members *B. cereus*, *B. mycoides*, and *B. thuringiensis* was shown. Restriction polymorphism patterns of the PCR products confirmed the presence of the NPRC gene in all three species.

Proteolytic bacteria are of central importance in nitrogen mineralization. There is evidence that proteases from *Bacillus cereus* and *B. mycoides*, which belong to the neutral metalloprotease class, play an important role in proteolytic processes in soils (6, 16–18). The DNA sequence of the *B. cereus* thermolysin-like enzyme has a high degree of homology to the sequences of other metalloproteases, especially those from *B. megaterium* and *B. stearothermophilus*. Moreover, the recently published DNA sequence of the gene for neutral protease, *nprA*, from *B. thuringiensis* (4) is nearly identical (97% homology on 945 bp).

The objective of this study was to develop primers and a functional probe for specific detection of the gene for neutral protease of *B. cereus*.

Type strains were purchased from the German Collection of Microorganisms and Cell Cultures. *B. cereus* and *B. mycoides* strains (Table 1) from four topsoils and three subsoils of different agricultural and forest sites at the Bornhöved Lake region in northern Germany were isolated on the gelatin liquefaction medium of Ewing (5) in March and October 1993. Strains CG 1 to CG 5 (Table 1) were isolated from a Canadian grassland rhizosphere soil. *B. cereus* 3045 was generously provided by the culture collection of the Department of Microbiology of the University of Guelph, Guelph, Ontario, Canada. Proteolytic bacteria were also isolated from a garden soil and a grassland rhizosphere soil in the area surrounding the GSF Research Center near Munich, Germany, and from an arable soil in Scheyern in south Germany (each from a 0- to 10-cm depth). Strains were obtained by plating soil suspensions on gelatin agar plates (2.5 g of Standard-I-Merck, 5.4 g of NaCl, 30 g of gelatin, and 12 g of agar, all per liter) and were investigated for morphological and physiological features according to classifications in *Bergey's Manual of Systematic Bacteriology* (7, 14). All strains were tested for gelatin liquefaction at 30°C for at least 1 week. *B. cereus* and *B. mycoides* were identified by their typical colony morphologies. Strains that did not form rhizoid colonies on agar were classified as *B. cereus*. Other *Bacillus* isolates were differentiated from *B. cereus* or *B. mycoides* but were not identified to the species level.

Strains were grown in 10 ml of nutrient broth for about 15 h at 30°C with shaking at 140 rpm. Total genomic DNA was isolated according to standard procedures (8).

The nucleotide sequence of the *B. cereus* neutral protease gene (NPRC) (19) was obtained from the National Center for Biotechnology Information data bank (accession no. M83910). The DNA region encoding the entire mature protein (951 bp) was used as the template for PCR, with upstream primer npr BcI (5′-GTAACAGGAACGAATAAAGTAGGAACTGGT AAAG-3′) and downstream primer npr BcII (5′-GTTTACAC CAACAGCACTAAATGATTGCTTAAC-3′). Amplification of DNA was carried out with the GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.). Samples of 50 μl contained 50 ng of template DNA, 25 pmol of each primer, 0.2 mM deoxynucleotide triphosphates (or 5 μl of PCR digoxigenin [DIG] mix [Boehringer, Mannheim, Germany] for generation of the probe), 2 U of Goldstar Red DNA polymerase, 5 μl of 10× reaction buffer (Eurogentec, Seraing, Belgium), and 3 mM $MgCl_2$. The PCR program was as follows: hot start cycle of 94°C for 5 min and 80°C for 4 min; one cycle of 94°C for 2 min, 64°C for 1 min, and 72°C for 2 min; 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. Amplified PCR products were analyzed by gel electrophoresis with 0.8% agarose in TAE buffer (40 mM Tris-acetate [pH 7.6], 1 mM Na_2EDTA). A DIG-labeled double-stranded DNA probe for NPRC was generated by PCR with the DIG DNA labeling kit (Boehringer) and the primers npr BcI and npr BcII; it comprised the whole sequence (951 bp) encoding the mature protein. Genomic DNA from *B. cereus* 3045 was used as the template.

For dot blot hybridization, 2.5 μg of genomic DNA was denatured in 250 μl of 0.4 N NaOH for 20 min and transferred to a positively charged nylon membrane (Boehringer) by vacuum blotting and subsequent baking at 120°C for 30 min. Hybridization with the NPRC probe and chemiluminescent detection were performed according to the protocol of Rost (11).

The accessibility of membrane-bound DNA of all of the tested bacteria to the NPRC probe was confirmed by their hybridization to the universal probe EUB 338 (1), with 16S ribosomal DNA as the target.

The PCR-amplified DNA was precipitated and resuspended in 50 μl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). Enzymatic digestion was performed in a mixture containing 8 μl of DNA solution, 5 U of enzyme (Boehringer), and 2 μl of the corresponding buffer, and sterile water was added to a total volume of 20 μl at 37°C overnight. After the addition of 0.5 μl

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TABLE 1. Characterization of the PCR-amplified genes of the *B. cereus* and *B. mycoides* isolates and of reference strains by restriction analysis

Source and isolate[a]	Species	Hybridization with NPRC probe	Restriction enzyme digestion (restriction site)				
			*Eco*RI (532)	*Hin*dIII (722)	*Sph*I (442)	*Hpa*I (434)	*Pst*I (882)
B. cereus DSM 3101^T		+	+	+	+	+	+
Arable field							
Ap 2 m'93	*B. cereus*	+	+	+	−	−	+
Ap 3 o'93	*B. cereus*	+	−	−	+	−	+
Ap 4 o'93	*B. mycoides*	+	+	+	−	−	+
Ap 5 o'93	*B. mycoides*	+	+	+	−	−	+
B 1 m'93	*B. cereus*	+	+	+	−	−	+
B 2 o'93	*B. cereus*	+	+	+	−	−	+
Grassland							
MAh 1 m'93	*B. cereus*	+	+	+	−	−	+
MAh 2 o'93	*B. cereus*	+	+	+	−	−	+
MAh 3 m'93	*B. mycoides*	+	+	+	−	−	+
MAh 4 o'93	*B. mycoides*	+	+	+	−	−	+
Wet grassland							
Aa 1 m'93	*B. cereus*	+	+	−	−	−	−
Aa 3 o'93	*B. cereus*	+	+	+	−	−	+
Aa 4 o'93	*B. mycoides*	+	+	+	−	−	+
Aa 5 m'93	*B. cereus*	+	+	+	−	−	+
Aa 6 o'93	*B. cereus*	+	+	+	−	−	+
Beech forest							
Ah 1 m'93	*B. cereus*	+	+	+	−	−	+
Ah 2 m'93	*B. cereus*	+	+	+	+	−	+
Ah 3 m'93	*B. mycoides*	+	+	+	−	−	+
B 1 m'93	*B. cereus*	+	+	+	−	−	+
B 2 m'93	*B. cereus*	+	+	+	+	−	+
Canadian grassland							
CG 1	*B. cereus*	+	+	+	+	−	+
CG 2	*B. cereus*	+	+	+	+	−	+
CG 3	*B. cereus*	+	+	+	+	−	+
CG 4	*B. cereus*	+	−	+	+	−	+
Reference strains							
B. cereus 3045[b]		+	+	+	+	−	+
B. mycoides DSM 2048^T		+	+	+	−	−	−
B. mycoides DSM 303		+	+	−	−	−	+
B. thuringiensis DSM 2046^T		+	+	+	+	−	+
CG 5[c]	*Bacillus* sp.	−					
B. megaterium DSM 32^T[c]		−					
B. megaterium DSM 90[c]		−					
B. stearothermophilus[c]		−					

[a] Ap, Ah, MAh, Aa, and B represent soil horizons; m'93 and o'93 indicate that samples were obtained in March 1993 and October 1993, respectively.
[b] Canadian isolate used for generation of probe.
[c] No PCR product was obtained.

of 10% sodium dodecyl sulfate, DNA digestion was analyzed by agarose gel electrophoresis.

Sixteen gram-negative and 30 gram-positive strains of proteolytic soil bacteria representing the culturable proteolytic populations of the three soils, mainly *Bacillus* species, various *Pseudomonas fluorescens* biotypes, and strains belonging to the *Flavobacterium-Cytophaga* group, were tested for probe specificity. The functional gene probe for NPRC hybridized to genomic DNA of all soil strains of *B. cereus* and *B. mycoides* under the chosen stringent conditions. No hybridization was obtained with genomic DNA of any gram-negative isolates or other *Bacillus* isolates (Fig. 1).

The probe hybridized to DNA from all the *B. cereus* and *B. mycoides* isolates from the different sites of and sampling times for the Bornhöved Lake region and the Canadian grassland rhizosphere soil (Table 1). Genomic DNA from the reference strains *B. mycoides* DSM 2048^T and *B. thuringiensis* DSM 2046^T also hybridized with the probe. DNA from *B. megaterium* DSM 32^T, *B. megaterium* DSM 90, and *B. stearothermophilus*, whose neutral protease genes showed 74% DNA sequence homology on 947 bp and 69% on 866 bp to that of *B. cereus*, was not detected. No further selectivity of hybridization could be obtained by applying more stringent conditions (data not shown).

Primers and PCR conditions were suitable for selective amplification of the protease genes of the type strains *B. cereus* DSM 3101, *B. mycoides* DSM 2048, and *B. thuringiensis* DSM 2046 and of representative soil isolates from the Bornhöved and Ontario sites (Table 1). PCR products were all of the expected sizes and were detected with the specific NPRC

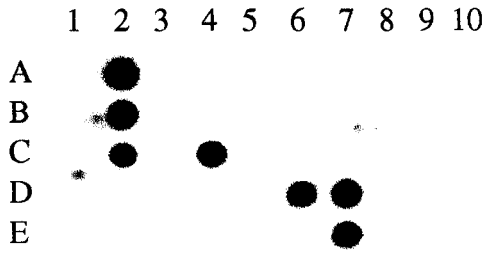

FIG. 1. Specificity of the functional NPRC probe was determined by dot blot hybridization with genomic DNA of proteolytic soil bacteria. Hybridization was achieved with *B. cereus* (A2, C4, and D7), *B. mycoides* (B2, C2, and D6), and *B. cereus* DSM 3101^T (E7). Hybridization was not achieved with *P. fluorescens* biotype I (A1, A3, C3, C7, D2, and D9); *P. fluorescens* biotype II (A6, A8, A9, C5, C9, D3, and E3); *Flavobacterium-Cytophaga* (B8, D4, and E6); *Bacillus* strains A (A4), B (A5), C (A7, B1, B4, C10, D1, D8, and E4), D (C6), E (B3), F (B5), G (C1), H (A10), I (B10), J (B9), K (B6), L (B7), M (C8 and E1), N (D5), and O (D10); coryneform X (E2); or coryneform Y (E5).

probe by Southern blot hybridization (data not shown). The protease genes of *B. megaterium* and *B. stearothermophilus* were not amplified by PCR. Sequence analysis revealed that their corresponding primer targets contain numerous mismatches, especially in the inner parts of the primer regions.

The PCR-amplified genes of the isolates were subjected to further characterization by restriction enzyme digestion (Table 1). The predicted restriction sites for the gene of *B. cereus* DSM 3101^T were confirmed. The specific restriction sites for *Eco*RI, *Hin*dIII, and *Pst*I were present in most of the bacterial isolates. Interestingly, the restriction site for *Hpa*I was not found in any soil bacterial isolate or in the reference strains. The restriction site for *Sph*I was rarely present in the German isolates but was found in all the Canadian isolates from the grassland rhizosphere samples and the Canadian strain *B. cereus* 3045, from which the probe had been generated. No clear distinctions between the *B. cereus* and *B. mycoides* isolates and the *B. thuringiensis* type strain could be made. The neutral proteases of strains Ap 3 o'93, Aa 1 m'93, Ah 2 m'93, and B 2 m'93 and the Canadian isolates may differ from the other neutral proteases in physical and biochemical properties since the variations in restriction pattern involve DNA regions responsible for the incorporation of amino acids involved in Zn^{2+} binding (*Sph*I), Ca^{2+} binding (*Eco*RI), and salt bridges (*Pst*I) (13).

Our results indicate that the gene for neutral protease has been highly conserved during the evolution of the *B. cereus* group species. These species (*B. cereus*, *B. mycoides*, *B. thuringiensis*, and *B. anthracis*) share phenotypic properties, and their status as separate species is still under debate. They have very high degrees of 16S rDNA sequence similarity as measured by 16S rRNA analysis (2), restriction fragment length polymorphism of rRNA genes (10), and analysis of intergenic spacers (3). Nakamura and Jackson (9) demonstrated that DNA-DNA relatedness between *B. cereus* and *B. mycoides* was 22 to 44% whereas that between *B. cereus* and *B. thuringiensis* was 59 to 69%, so they concluded that *B. cereus* and *B. thuringiensis* are genetically related but taxonomically distinct entities. The authors classified *B. mycoides* as genetically distantly related to *B. cereus*. Seki et al. (12) showed DNA homologies of 54 to 80% between *B. cereus* and *B. thuringiensis* and postulated that these organisms should be considered to belong to one species. In the investigations of Somerville and Jones (15), no clear distinctions were evident between *B. cereus*, *B. thuringiensis*, and *B. anthracis* in DNA-DNA hybridization with *B. cereus* as a reference organism.

Many strains which appeared to be *B. cereus* phenotypically have been shown by fatty acid analysis and by DNA-DNA hybridization to be *B. mycoides* strains (20). Nevertheless, our approach is qualified for the detection of the gene for one neutral protease which is potentially expressed by *B. cereus*, *B. mycoides*, and *B. thuringiensis* in soils. Primers and the NPRC functional probe can now be used to investigate the expression of the neutral protease of the *B. cereus* group members in soils at the mRNA level. This would reveal further information about the regulation of nitrogen mineralization in soils.

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