

# PCR primers and functional probes for amplification and detection of bacterial genes for extracellular peptidases in single strains and in soil

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## Abstract

A set of primers and functional probes was developed for the detection of peptidase gene fragments of proteolytic bacteria. Based on DNA sequence data, degenerate PCR primers and internal DIG-labeled probes specific for genes encoding alkaline metallopeptidases (*apr*) (E.3.4.24), neutral metallopeptidases (*npr*) (E.3.4.24) and serine peptidases (*sub*) (E.3.4.21) were derived by multiple sequence alignments.

Type strains with known peptidase genes and proteolytic bacteria from a grassland rhizosphere soil, a garden soil and an arable field were investigated for their genotypic proteolytic potential. For 52 out of 53 proteolytic bacterial isolates, at least one of the three peptidase classes could be identified by this approach. The amplified gene fragments were of the expected sizes with each of the three primer sets. The functional probes APR, NPR and SUB have been shown to hybridize specifically to the corresponding gene fragments. *sub* and *npr* genes were mainly found in *Bacillus* species. *apr* genes were only found in the *Pseudomonas fluorescens* biotypes and in two morphologically identical *Flavobacterium–Cytophaga* strains from two different sites. In most of the *Bacillus* spp., both *sub* and the *npr* and in the *Flavobacterium–Cytophaga* strains even all the three genes could be detected. PCR with DNA isolated from soil led to one main product of the expected size with each primer pair whose identity was additionally confirmed by Southern blot hybridization with the corresponding probes. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Genes for extracellular proteases; Metallopeptidases; N-mineralization; Primers and probes; Serine peptidases; Soil bacteria

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## 1. Introduction

Hydrolysis of peptidic compounds by extracellular microbial peptidases is of key importance for the mobilization of ammonium and subsequent nitrogen

cycling processes in ecosystems. The potential for degradation of diverse nitrogenous compounds depends on the equipment of the indigenous microbial community with genes encoding the required enzymes. Only limited information is available about the sources and the nature of soil peptidases. By cultivation methods, *Pseudomonas fluorescens* biotypes, *Bacillus cereus* and *B. mycoides*, and *Flavobacterium–Cytophaga* species were found to be

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numerically dominant proteolytic bacteria in a broad range of different soils, all excreting metallopeptidases during culture (Bach and Munch, 2000). Watanabe and Hayano (1993a,b, 1994) have shown, that *Bacillus* peptidases, and especially the neutral metallopeptidase of *B. cereus* and *B. mycoides* and the *B. subtilis* like alkaline serine peptidase may be regarded as the predominant enzymes active in peptidic degradation in paddy field sites. The few investigations on peptidase sources in soils are all based on cultivation techniques and on the comparison of the highly complex peptidase mixtures in soils with pure peptidases of culturable bacteria. Investigations at the soil DNA, or even mRNA level, could reveal new and more detailed information about the structure and dynamic of proteolytic bacterial communities and their actual impact in nitrogen turnover in ecosystems independent on culturability.

It is presently known that the predominant extracellular peptidases of fungal origin are supposedly cysteine and aspartic peptidases, whereas those of bacterial origin are mainly alkaline metallopeptidases (Apr), neutral metallopeptidases (Npr) and serine peptidases (Sub) (Kalisz, 1988). Alignments of DNA sequences encoding the latter enzymes should reveal conserved regions in each peptidase class which would allow the design of degenerate primers and probes located in the inner part of the target sequences. In the present study, we have developed and tested PCR primers and probes as potential tools for the detection and identification of peptidase genes in proteolytic bacteria and for the study of peptidase specific genetic diversity and gene expression of proteolytic decomposer communities independent of culturability.

## 2. Material and methods

### 2.1. Strains and culture conditions

Type strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Proteolytic soil bacteria (Table 3) from a grassland rhizosphere, a garden soil and an arable soil were isolated and identified as described in Bach

et al. (1999a). The methods used for the characterization of the bacteria are described in Starr et al. (1981). The *P. fluorescens* group included different biotypes (as indicated by different colony morphologies) which were not further characterized. The identity of the *Pseudomonas* spp. was confirmed by PCR using primers specific for *Pseudomonas sensu stricto* (Widmer et al., 1998). Strains belonging to the *Flavobacterium–Cytophaga* complex were assigned to this group when hybridization with the 16S rRNA-specific probe (CF319a) for bacteria of the phylum cytophaga–flavobacter–bacteroides was observed (Manz et al., 1996). The strains S 51 and Gs 61 had identical colony morphologies. The identification of *B. cereus* and *B. mycoides* strains is described in Bach and Munch (2000). *Bacillus* spp. other than *B. cereus* and *B. mycoides* were tested for formation of endospores and not further characterized. Different colony morphologies are indicated by different letters from A to O.

Strains were grown in 10 ml of nutrient broth medium (Merck, Darmstadt, Germany) at 30°C, except *B. licheniformis*, *B. stearothermophilus* and *B. caldolyticus* which were incubated at 37°C, 50°C and 70°C, respectively, with shaking at 130 rpm over night.

### 2.2. Extraction of DNA

Genomic DNA of pure bacterial cultures was obtained by standard procedures for DNA extraction (Marmur, 1961). Soil DNA from an arable field (Scheyern, southern Bavaria, Germany) was extracted and purified by using the FastDNA SPIN Kit for Soil (Bio 101, Vista, USA) as recommended by the manufacturer. Soil and site characteristics: HK—high yield area with conventional land farming, sandy silty loam, pH 5.6, 16.21 mg · g<sup>-1</sup> organic C, 1.54 mg · g<sup>-1</sup> total N.

### 2.3. Design of PCR primers and probes

Research for DNA sequences of bacterial peptidases in the NCBI database revealed several main groups: the thermolysin-like *Bacillus* neutral metallopeptidases (Npr), the alkaline metallopeptidases of

Table 1  
Main extracellular bacterial endopeptidases

Catalytic type	Subgroup		Groups of bacteria in which producers are found and from which gene sequences are available in NCBI database	Sequences considered in alignments and detected by the oligonucleotides presented in this study
Serine peptidases	Serine alkaline peptidases; subtilisin-like peptidases		CFB-group; alpha and gamma-Proteobacteria; <i>Bacillus</i> / <i>Clostridium</i> group; <i>Streptomyces</i> spp.	none
	Subtilisins	Carlsberg, BPN	<i>Bacillus</i> spp.	( <i>sub</i> ) <i>B. licheniformis</i> (S78160), <i>B. amyloliquefaciens</i> (K02496), <i>B. subtilis</i> (S51909), <i>Bacillus</i> sp. (D29736), <i>Bacillus</i> sp. (U39230)
Cysteine peptidases Metallo-peptidases	neutral	thermolysin-like	<i>Porphyromonas gingivalis</i> ; <i>Streptococcus pyogenes</i> .	not investigated
			<i>Bacillus</i> ; <i>Streptococcus</i> spp.; <i>Bacteroides</i> ; <i>Erwinia chrysanthemi</i> ; <i>Serratia marcescens</i> ; <i>Vibrio</i> spp.; <i>B. brevis</i> <i>B. subtilis</i>	( <i>npr</i> ) <i>B. megaterium</i> (X75070), <i>Paenibacillus polymyxa</i> (D00861), <i>Lactobacillus</i> sp. (D29673), <i>B. stearothermophilus</i> (M11446), <i>B. caldolyticus</i> (U25629), <i>B. cereus</i> (M83910), <i>B. thuringiensis</i> (L77763), <i>Alicyclobacillus acidocaldarius</i> (U07824), <i>Staphylococcus epidermis</i> (X69957), <i>B. thermoproteolyticus</i> (X76986), <i>B. amyloliquefaciens</i> (K02497), <i>B. brevis</i> (X61286), <i>Clostridium perfringens</i> (D45904), <i>Listeria monocytogenes</i> (X54619)
		collagenase	CFB-group; <i>Clostridium</i> spp.; <i>Vibrio</i> spp.; <i>B. cereus</i>	none
		elastase	<i>P. aeruginosa</i> ; <i>Aeromonas</i> spp., <i>Vibrio</i> spp.	none
	alkaline		gamma-proteobacteria	( <i>apr</i> ) <i>Pseudomonas fluorescens</i> (ab013895), <i>P. tolaasii</i> (AJ007827), <i>P. aeruginosa</i> (D87921), <i>Pseudomonas</i> sp. (Y17314), <i>Erwinia chrysanthemi</i> (M60395), <i>Serratia marcescens</i> (X55521), <i>Serratia</i> sp. (X04127)

The classification has been summarized from that of Rao et al. (1998). A detailed classification of proteolytic enzymes is given by Barrett (1994, 1995).

gram (–) bacteria (*Apr*) and the subtilisins of *Bacillus* species (*Sub*). The nucleotide sequences of the mature peptidases were considered for homology search using the NCBI BLASTN program. Genes with homologous regions either to the *apr* gene of *P. fluorescens*, to the *npr* gene of *B. cereus* or to the *sub* gene of *B. subtilis* were extracted from the NCBI database (Table 1). Alignments were performed using the Genomatix DiAlign program (<http://genomatix.gsf.de/cgi-bin/dialign/dialign.pl>). Primer target regions were chosen to span DNAD fragments of about 300 bp or less. The probe regions were located to the inner part of the amplicons. Comparison of the designed degenerate oligonucleotides to known DNA sequences (EMBL, Release 62) using the Genomatix Matinspector program (<http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl>) revealed that the detection system would be specific for bacterial peptidase genes and discriminate fungal peptidase genes. Table 1 reviews the main bacterial extracellular endopeptidase groups and indicates, which groups of peptidases are investigated and from which organisms sequences were considered in the alignments. The deviated oligonucleotides are described in Table 2.

#### 2.4. PCR

Amplification was carried out with the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT,

USA). DNA from bacterial cultures: 50  $\mu$ l volumes containing 50 ng of template DNA, primer (75 pmol of each sub Ia or Ib/II; 50 pmol of npr I/II and apr I/II), 0.2 mM of deoxynucleotide triphosphates, 5  $\mu$ l of 10  $\times$  reaction buffer, 3 mM of MgCl<sub>2</sub> and 1 U of Goldstar “Red” DNA Polymerase (Eurogentec, Seraing, Belgium). The PCR program was as follows: hot start cycle of 95°C for 5 min, 80°C for 5 min (polymerase was added during this step); 30 cycles of 94°C for 30 s, 53°C or 49°C for 30 s, and 72°C for 20 s; final extension at 72°C for 10 min. DNA from soil: when soil DNA was used as template, 2  $\mu$ l of the 50  $\mu$ l DNA eluate obtained from 500 mg of soil was applied. Seventy-five picomole of the primers sub Ia/II and npr I/II and 50 pmol of apr I/II were used. BSA was added to the reaction mix to a final concentration of 0.3%. DMSO was added to a final concentration of 5% when PCR with the primers apr I/II was performed. Since all the three assays were highly sensitive to the amount of polymerase, the polymerase solution was diluted in 1  $\times$  reaction buffer in order to increase the added volume to 2  $\mu$ l. For the amplification of *apr*, 1 U was applied, and 2 U for each *npr* and *sub*. The amount of MgCl<sub>2</sub> was 1.5 mM for *npr* and *apr* and 3 mM for *sub*. The PCR program was the same as for genomic DNA from isolates, except that annealing temperatures were 55°C for all the three PCRs. Amplified PCR products were analyzed by gel electrophoresis with 1.8% agarose in 1  $\times$  TAE buffer (40

Table 2

Oligonucleotides used as primers and probes for specific amplification and detection of genes for alkaline metallopeptidases (*apr*), neutral metallopeptidases (*npr*) and serine peptidases (*sub*)

Oligonucleotide	Composition	Position (nt) <sup>a</sup>	T <sub>m</sub> (2AT + 4GC) (°C)	Length of amplicon (bp)
FP <i>apr</i> I	5'-TAYGGBTTCAAYTCCAAYAC-3'	808–827	52–60	194
probe APR	DIG-5'-ARCCVAGAGAARTCVARGGTRTC-3'	901–922	60–68	
RP <i>apr</i> II	5'-VCGGATSGAMACRTRCC-3'	985–1002	52–60	233
FP <i>npr</i> I	5'-GTDGAYGCHCAYTAYTAYGC-3'	214–233	54–66	
probe NPR	DIG-5'-TAHAYCATYTGKADCCRTTCCA-3'	346–368	58–70	
RP <i>npr</i> II	5'-ACMGCATGBGTYADYTCATG-3'	437–446	54–66	
FP <i>sub</i> Ia	5'-ATGSAYRTRYAAAYATGAG-3'	853–872	50–56	319
FP <i>sub</i> Ib	5'-GNACHCAYGTDGCHGGHAC-3'	692–710	58–62	
probe SUB	DIG-5'-TTGAHRTYDYKGCWCCWGGY-3'	1085–1004	56–62	486
RP <i>sub</i> II	5'-GWGWHGCCATNGAYGTWC-3'	1154–1171	52–58	

<sup>a</sup>Nucleotide position in the *apr* gene of *P. fluorescens* (NCBI AB013895), in the *npr* gene of *B. cereus* (NCBI M38910) and in the *sub* gene of *B. subtilis* (NCBI S51909).

mM Tris–HCl [pH 7.6], 20 mM acetic acid, 1 mM Na<sub>2</sub>EDTA).

### 2.5. Probe hybridization

For dot blot hybridizations on positively charged nylon membranes (Boehringer, Mannheim, Germany), 4–12 µl of PCR product were denatured in 250 µl of 0.4 N NaOH for 20 min and vacuum blotted. Southern transfer was performed as described by the manufacturer (Boehringer, Mannheim, Germany). DNA was fixed to the membrane by UV-cross-linking. Hybridization with the probes NPR, APR and SUB (Table 2) and chemiluminescent detection was performed as follows: formamide concentrations in the prehybridization solution was 5% for NPR and APR, and 0% for SUB. Prehybridization in 5 × SSC; 0.1% N-lauroylsarcosine; 0.02% SDS; 1% blocking reagent and formamide for 1.5 h at 45°C. Hybridization with 10 pmol/ml of 5′ DIG-labeled probe in prehybridization solution for 2.5 h at 45°C. Washings were performed 2 × 5 min with 2 × SSC, 0.1% SDS at room temperature and 2 × 15 min in 0.5 × SSC, 0.1% SDS at 45°C. Detection was performed by using the DIG Luminescent Detection Kit (Boehringer Mannheim, Germany) as recommended by the manufacturer.

## 3. Results and discussion

### 3.1. Validation of the designed oligonucleotides

The applicability of the developed primer pairs and DIG-labeled oligonucleotide probes to detect the corresponding peptidase genes by PCR and subsequent dot blot hybridization was confirmed for all the selected representative strains of the three different alignment groups (highlighted in bold face in Table 3), except for *npr* of *B. amyloliquefaciens*. The annealing temperature of 53°C was suitable for the generation of specific amplicons of the expected lengths of most of these genes in all three PCR reactions. Lowering the annealing temperatures (49°C or 43°C), led to the appearance of additional multiple unspecific bands, but also to the detection of addi-

tional genes, such as *npr* in *B. subtilis* and *B. licheniformis*. Southern blot hybridization with the SUB probe revealed PCR products from *B. cereus* and *B. thuringiensis*, generated with the primers sub Ia/II (data not shown). The *sub* gene of *B. megaterium* could be amplified when the forward primer sub Ia was replaced by sub Ib, generating a band of 486 bp length. The reported *sub* gene of *B. stearothersophilus* (Jang et al., 1992) was not detectable when applying these PCR conditions. *Escherichia coli* and *P. chlororaphis* were shown to harbour *npr* and *apr* genes, respectively. All the amplified gene fragments were detected specifically by dot blot or Southern blot hybridization using the corresponding DIG-labeled oligonucleotide probes (Table 3).

### 3.2. Proteolytic soil bacteria

The proteolytic soil bacterial isolates investigated in this study are also presented in Table 3. Under the applied cultivation conditions, *B. cereus*, *B. mycoides* and *P. fluorescens* biotypes I and II appeared to be the most abundant culturable species in the garden soil, the grassland rhizosphere soil and in the arable soil (data not shown). The other strains were not identified to the species level, but most were assigned to the genus *Bacillus* and some to the *Flavobacterium–Cytophaga* group. The strains S 28, Rh 9 and S 21 were not further characterized.

### 3.3. Detection of peptidase genes

For each investigated soil isolate, except for S 21, gene fragments of at least one peptidase class could be detected. In 17 out of 22 morphologically different isolates (*B. cereus*, *B. mycoides*, *Bacillus* sp. A, B, C, D, E, F, G, H, L, M, N, and O, the *Flavobacterium–Cytophaga* strains and the unidentified strains S 28 and Rh 9), the *npr* gene could be identified by PCR using the primers *npr* I/II and by subsequent dot blot hybridization with the probe NPR. The presence of the *npr* genes in the *B. cereus* group members (*B. cereus*, *B. mycoides* and *B. thuringiensis*) is in agreement with our recent findings that the whole DNA sequence encoding the mature neutral

Table 3  
 Characterization of peptidase genes of type strains and soil bacterial isolates

Strain	Reported genes	PCR amplification with primers/hybridization with probes		
		apr I/II	sub Ia(Ib)/II	npr I/II
<i>B. cereus</i> DSM 3101 <sup>T</sup>	<i>npr</i> <sup>a</sup>	–	Ia + <sup>b</sup> /SUB	+ /NPR
<i>B. thuringiensis</i> DSM 2046 <sup>T</sup>	<i>sub</i> / <i>npr</i> <sup>a</sup>	–	Ia + <sup>b</sup> /SUB	+ /NPR
<i>B. megaterium</i> DSM 32 <sup>T</sup>	<i>npr</i> <sup>a</sup>	–	Ib + <sup>c</sup> /SUB	+ /NPR
<i>B. stearothersophilus</i> DSM 22 <sup>T</sup>	<i>sub</i> / <i>npr</i> <sup>a</sup>	–	–	+ /NPR
<i>Paenibacillus polymyxa</i> DSM 36 <sup>T</sup>	<i>npr</i> <sup>a</sup>	–	–	+ <sup>c</sup> /NPR
<i>B. amyloliquefaciens</i> DSM 7 <sup>T</sup>	<i>sub</i> <sup>a</sup> / <i>npr</i> <sup>a</sup>	–	Ia + /SUB	–
<i>B. caldolyticus</i> DSM 405	<i>npr</i> <sup>a</sup>	–	–	+ /NPR
<i>B. mycooides</i> DSM 2048 <sup>T</sup>	<i>npr</i> <sup>a</sup>	–	–	+ /NPR
<i>E. coli</i> DSM 30083 <sup>T</sup>	<i>sub</i>	–	–	+ /NPR
<i>B. firmus</i> DSM 12 <sup>T</sup>	?	–	–	+ <sup>c</sup> /NPR
<i>P. aeruginosa</i> DSM 50071 <sup>T</sup>	<i>apr</i> <sup>a</sup>	+ /APR	–	–
<i>P. fluorescens</i> DSM 50090 <sup>T</sup>	<i>apr</i> <sup>a</sup>	+ /APR	–	–
<i>S. marcescens</i> DSM 30121 <sup>T</sup>	<i>apr</i> <sup>a</sup>	+ /APR	–	–
<i>Erwinia chrysanthemi</i> DSM 4610 <sup>T</sup>	<i>apr</i> <sup>a</sup>	+ /APR	–	–
<i>P. chlororaphis</i> DSM 50083 <sup>T</sup>	?	+ /APR	–	–
<i>B. subtilis</i> DSM 22 <sup>T</sup>	<i>npr</i> / <i>sub</i> <sup>a</sup>	–	Ia + /SUB	+ <sup>b</sup> /NPR
<i>B. licheniformis</i> DSM 13 <sup>T</sup>	<i>sub</i> <sup>a</sup>	–	Ia + /SUB	+ <sup>b</sup> /NPR
<i>B. cereus</i> (Rh) 5	<i>npr</i>	–	Ia + <sup>c</sup> /SUB	+ /NPR
<i>B. mycooides</i> 1	<i>npr</i>	–	Ib + <sup>c</sup> /SUB	+ <sup>c</sup> /NPR
<i>B. cereus</i> (Gs) 6	<i>npr</i>	–	Ib + <sup>c</sup> /SUB	+ /NPR
<i>B. mycooides</i> (Gs) 28	<i>npr</i>	–	–	+ /NPR
<i>B. cereus</i> (S) 4	<i>npr</i>	–	–	+ /NPR
<i>B. mycooides</i> (S) 3	<i>npr</i>	–	–	+ /NPR
<i>Bacillus</i> sp. A (Gs) 11	?	–	Ia + <sup>c</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. B (Gs) 13	?	–	Ia + <sup>c</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. C (Gs) 25	?	–	Ia + <sup>c</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. C (Rh) 33	?	–	Ia + <sup>c</sup> /SUB	+ <sup>c</sup> /NPR
<i>Bacillus</i> sp. C (S) 5	?	–	Ib + <sup>c</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. D (S) 70	?	–	Ia + /SUB	+ /NPR
<i>Bacillus</i> sp. E (Gs) 31	?	–	Ia + <sup>c</sup> /SUB <sup>d</sup>	+ <sup>b</sup> /NPR <sup>d</sup>
<i>Bacillus</i> sp. F (Gs) 40	?	–	Ia + <sup>c</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. G (Gs) 66	?	–	Ia + /SUB	+ <sup>b</sup> /NPR
<i>Bacillus</i> sp. H (Gs) 21	?	–	–	+ /NPR
<i>Bacillus</i> sp. I (Gs) 64	?	–	Ia + /SUB	–
<i>Bacillus</i> sp. J (Gs) 62	?	–	Ia + /SUB	–
<i>Bacillus</i> sp. K (Gs) 41	?	–	Ia + /SUB	–
<i>Bacillus</i> sp. L (Gs) 42	?	–	Ia + <sup>c</sup> /SUB	+ <sup>b</sup> /NPR
<i>Bacillus</i> sp. M (S) 20	?	–	Ia + <sup>b</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. M (Rh) 20	?	–	Ia + <sup>b</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. N (S) 1	?	–	Ia + <sup>b</sup> /SUB	+ /NPR
<i>Bacillus</i> sp. O (S) 16	?	–	–	+ /NPR
<i>P. fluorescens</i> I (S) 6	<i>apr</i>	+ /APR	–	–
<i>P. fluorescens</i> II (S) 22	<i>apr</i>	+ /APR	–	–
<i>P. fluorescens</i> I (Rh) 2	<i>apr</i>	+ /APR	–	–
<i>P. fluorescens</i> II (Gs) 3	<i>apr</i>	+ /APR	–	–
<i>P. fluorescens</i> I (Gs) 9	<i>apr</i>	+ /APR	–	–
<i>Flavobacterium</i> – <i>Cytophaga</i> (S) 51	?	+ <sup>c</sup> /APR	Ia, Ib + / –	+ /NPR
<i>Flavobacterium</i> – <i>Cytophaga</i> (Gs) 61	?	+ <sup>c</sup> /APR	Ia, Ib + / –	+ /NPR
Gram (–) motile rods (S) 28	?	–	Ia + <sup>b</sup> /SUB	+ /NPR
Gram (+) rods (Rh) 9	?	–	Ia + <sup>b</sup> /SUB	+ /NPR
Gram (+) coryneform (S) 21	?	–	–	–

protease of *B. cereus* is highly conserved within those species (Bach et al., 1999a).

The *sub* gene was amplified by PCR for 17 different isolates, mostly *Bacillus* species (*B. cereus*, *B. mycoides*, *Bacillus* sp. A, B, C, D, E, F, G, I, J, K, L, M, N) and the *Flavobacterium–Cytophaga* species. The amplicons of the *Bacillus* species were detectable with the SUB probe whereas those of the *Flavobacterium–Cytophaga* species were not. The presence of this gene in the latter species was therefore confirmed by PCR using the second forward primer sub Ib, which determines a fragment size of 486 bp. Results were not consistent for the *B. cereus* and *B. mycoides* isolates, since *sub* genes were not detected for the isolates Gs 28, S 3 and S 4. For isolates Rh 1 and Gs 6, the sub Ib forward primer had to be used and for Rh 5 and the type strains *B. cereus* and *B. mycoides* lower annealing temperatures had to be applied. The SUB probe hybridized specifically to these generated amplicons. These results suggest that the *sub* gene, like the *npr* gene, is also present in the three *B. cereus* group species. Analysis of the DNA sequence of the alkaline protease of *B. thuringiensis* (AF170567), which first was not included into the alignments for *sub*, showed that only the reverse primer sub II matches to 100%. Sequencing of the PCR products obtained by applying primer sub Ib could allow the consideration of the sub Ia target region in the design of more appropriate primers.

The presence of both genes (*npr* and *sub*) in almost all *Bacillus* species is not unusual and has been reported for several *Bacillus* species, such as *B. thuringiensis*, *B. stearothermophilus*, *B. amyloliquefaciens* and *B. subtilis* (Jang et al., 1992; Millet, 1969).

The *apr* gene was only demonstrated for the *P. fluorescens* biotypes of all three sites and for the *Flavobacterium–Cytophaga* strains of the arable field and the garden soil.

The presence of all the three genes in the *Flavobacterium–Cytophaga* bacteria was not expected because the designed oligonucleotides did not match to any known DNA sequence of such bacteria using the Genomatix MatInspector program even allowing three mismatches to the degenerate primers as well as the probe. The presence of diverse classes of peptidase genes matches with the putative ecological importance of these organisms in the degradation of complex polymeric compounds in soils (Reichenbach and Dworkin, 1981).

None of the peptidase genes could be demonstrated for the coryneform strain S 21. This result agrees with the fact that none of the oligonucleotides used in this study matched with any peptidases of *Actinomycetales* members in the database and that initial homology research revealed conserved DNA regions among *Streptomyces* peptidases, different from those investigated in this study (data not shown).

There is only few indication for sequence homology of bacterial exopeptidase genes with fungal ones as far as sequences are known. Except for sub II, none of the presented oligonucleotides nor whole sequences encoding the mature bacterial enzymes are matching with any fungal sequences in computational homology research (as indicated in Section 2), even if manifold sequences of fungal serine and metallopeptidases are available. The target region of primer sub II is found in some fungal species, but with at least one mismatch. So, the amplification of one of the peptidase gene fragments presented in this study from fungal DNA cannot be expected.

### 3.4. Restrictions

Since only the genes with consensus DNA regions could be considered, not all soil bacterial peptidase genes are covered by the introduced primers and probes. As for serine peptidases, the serine alkaline and the subtilisin-like enzymes are excluded from

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#### Notes to Table 3:

Rh = grassland rhizosphere soil; Gs = garden soil; S = arable soil (Scheyern).

<sup>a</sup>DNA sequence was considered in multiple alignments.

<sup>b</sup>Amplification was achieved only at 43°C annealing temperature.

<sup>c</sup>Amplification was achieved only at 49°C annealing temperature.

<sup>d</sup>Weak dot blot hybridization signal.

detection (Table 1). In sequence analysis, these genes did not show any conserved DNA regions, which would allow the development of appropriate oligonucleotides, except of several genes for alkaline serine peptidases of *Streptomyces* spp., which were different from those of the subtilisins. The sparse sequences of cysteine peptidases available in the database are those of clinical species and neither could be considered in this investigation. Nevertheless, specific research could reveal that this type of enzymes is more wide spread among soil bacteria

than so far assumed. The metallopeptidases collagenase and elastase, which are reported for several common soil bacteria are also excluded from detection by the presented approach. Even if the *P. aeruginosa* elastase shows amino acid homology regions that include structurally and functionally important residues of the *B. subtilis* thermolysin (Fukushima et al., 1989), no homology regions could be detected at the DNA sequence level.

We also point out that some sequences of the investigated groups did not fit into the alignments or would have caused even higher primer degeneracy, like the *npr* of *B. brevis*, *B. subtilis*, and *Serratia marcescens*. On the other hand, the *B. subtilis* *npr* gene was detected by the oligonucleotides when lowering the annealing temperature during PCR. The high diversity of ecological niches inhabited by proteolytic bacteria, the different functions of their peptidases and the high diversity of potential substrates probably have induced a strong variability of the corresponding genes.

### 3.5. Peptidase genes in soil

The oligonucleotides were applied for the detection of peptidase genes in total DNA isolated from soil. All the three genes, *apr*, *npr* and *sub* could be specifically amplified from soil DNA and were detected with the corresponding probes as shown by Southern blot hybridization (Fig. 1B, 1–3). A cycle number of 35 was sufficient to obtain clearly visible bands on agarose gel after ethidium bromid staining. For all the three PCR primer sets, the application of different annealing temperatures revealed 55°C to be optimal in terms of product yield and specificity of the PCR product.

## 4. Conclusion

We think that the use of these oligonucleotides covers a representative, although selective, part of the proteolytic soil bacterial community because *B. cereus*, *B. mycooides* and *P. fluorescens* (Bach and Munch, 2000) and their excreted peptidases (Hayano et al., 1987; Watanabe and Hayano, 1993a,b, 1994) have been shown to be significant in protein degradation in soils. In addition, we have shown that the

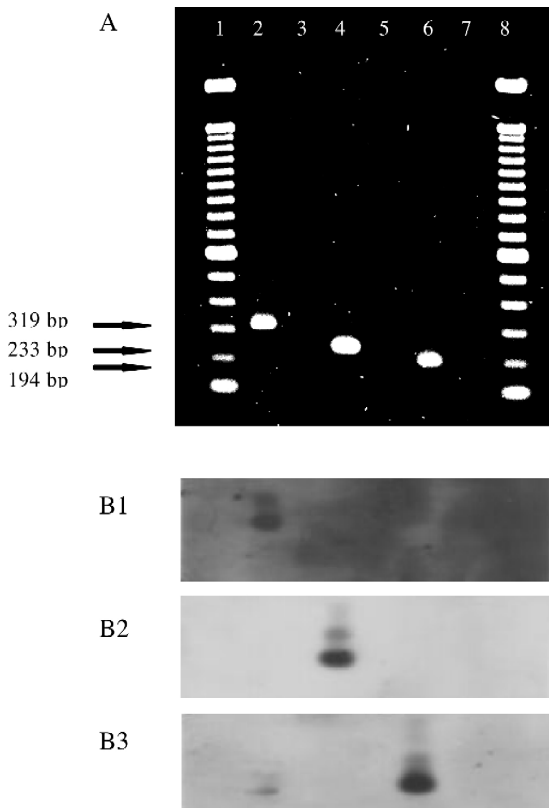


Fig. 1. (A) Gel electrophoresis of PCR products obtained from soil DNA with the primers specific for the genes for bacterial serine peptidases (*sub*), neutral metallopeptidases (*npr*) and alkaline metallopeptidases (*apr*). DNA size standard (lanes 1 and 8), amplification product with primers sub I a/sub II with DNA (lane 2) and without DNA (lane 3), amplification product with primers npr I/npr II with DNA (lane 4) and without DNA (lane 5), amplification product with primers apr I/apr II with DNA (lane 6) and without DNA (lane 7). (B) Southern blot hybridization of the gene fragments *sub*, *npr*, *apr* to the DIG-labeled probes SUB (B1), NPR (B2) and APR (B3).

oligonucleotides are suitable to detect previously unknown and even several different peptidase genes in almost all the bacterial proteolytic soil isolates obtained. The presented approach covers at least the fraction of bacteria obtained in cultivation-based studies and further investigations might show if an unculturable part of the proteolytic community is included.

A multiplex PCR approach has not been followed in this study because of the very high degeneracy of the primers. This assay would mean the presence of 608 different primers in one PCR reaction. As it was our goal to amplify as much genes as possible out of a highly complex mixture of DNA, we decided to work under optimal conditions for each peptidase class. This is supported by the fact that optimized conditions for the three PCR approaches differ in polymerase-,  $MgCl_2$ - or DMSO-concentration.

The soil target peptidase gene fragments may be amplified by PCR and investigated by sequence based separation techniques like DGGE or SSCP to create peptidase specific community patterns. The probes introduced in this study would also be appropriate for a sequence specific extraction of mRNA as we have previously shown for the mRNA for the *B. cereus* neutral protease (Bach et al., 1999b). The application of these tools would provide important information about the peptidase specific genetic potential of the soil bacterial community and its impact on nitrogen turnover.

Extracellular bacterial peptidases, and especially the thermostables, are also of commercial interest, like the subtilisins of *Bacillus* species, which are, e.g. used in detergents (Kalisz, 1988), the *P. fluorescens* peptidase involved in food spoilage (Alichanidis and Andrews, 1977), or the peptidase of *Lactobacillus helveticus* in the refinement of milk products (Chen and Steele, 1998). Thus, a screening for new peptidases with new or improved properties by using the presented tools may appear promising.

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