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Cloning of two pectate lyase genes from the marine Antarctic bacterium

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Abstract A marine Antarctic psychrotolerant bacterium (strain ANT/505), isolated from sea ice-covered surface water from the Southern Ocean, showed pectinolytic activity on citrus pectin agar. The sequencing of the 16S rRNA of isolate ANT/505 indicates a taxonomic affiliation to *Pseudoalteromonas haloplanktis*. The supernatant of this strain showed three different pectinolytic activities after growth on citrus pectin. By activity screening of a genomic DNA library of isolate ANT/505 in *Escherichia coli*, two different pectinolytic clones could be isolated. Subcloning and sequencing revealed two open reading frames (ORF) of 1,671 and 1,968 nt, corresponding to proteins of 68 and 75 kDa, respectively. The deduced amino acid sequence of the two ORFs showed homology to pectate lyases from *Erwinia chrysanthemi* and *Aspergillus nidulans*. The pectate lyases contain signal peptides of 17 and 26 amino acids that were correctly processed after overexpression in *E. coli* BL21. Both enzymes were purified by anionic exchange chromatography. Maximal enzymatic activities for both pectate lyases were observed at 30°C and a pH range of 9 to 10. The K_m values of both lyases for pectate and citrus pectin were 1 g l^{-1} and 5 g l^{-1} , respectively. Calcium was required for activity on pectic substrates, whereas the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) resulted in complete inhibition of the enzymes. These two

enzymes represent the first pectate lyases isolated and characterized from a cold-adapted marine bacterium.

Key words Pectate lyase · Pectin · Psychrophilic · Marine · *Pseudoalteromonas haloplanktis*

Introduction

The marine phytoplankton of permanently cold environments produces a variety of structural polymers such as cellulose and xylan. Although relatively few data are available concerning the hydrolysis of such compounds under low-temperature conditions, it is obvious that these structural polymers can be degraded by marine microorganisms. Cummings and Black (1999) reported screening for xylan- and cellulose-degrading microorganisms from the 3°C cold marine sediment of shallow waters around Adelaide Island, British Antarctica. They isolated a xylanolytic gram-negative bacterium with an optimal growth rate at 15°C.

Pectin, the important structural constituent of plant cell walls, is composed essentially of long chains of (1,4)- α -D-polygalacturonate, which are partially methyl esterified. Although pectin is of secondary importance for the marine environment, it is supposed that this polymer is also degraded by marine microorganisms (Cummings and Black 1999). Microbial pectin degradation is accomplished by methylesterases, which remove the methyl groups from pectin, and the depolymerases, which degrade both pectin and pectate (Whitaker 1991). Bacteria produce mainly pectate lyases, which require an alkaline pH and Ca^{2+} for their optimal enzyme activity. Reports on pectinases from cold-adapted microorganisms are so far restricted to psychrotolerant spoilage bacteria such as different strains of *Pseudomonas fluorescens* (Schlemmer et al. 1987). Recently, the production of pectate lyases by the psychrotolerant bacterium *Chryseomonas luteola* has been described (Laurent et al. 2000). This bacterium, which has been isolated from spoiled celeriac, is able to macerate

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plant tissue at low temperatures by means of at least three extracellular pectate lyases.

We report here the isolation of a pectinolytic marine bacterium from Antarctic sea ice. This psychrotolerant marine bacterium produces at least two pectate lyases. The genes of both enzymes were cloned and sequenced, and the pectate lyases were purified and characterized.

Materials and methods

Strains and cultivation conditions

The strain ANT/505 has been isolated from sea ice-covered surface water collected in the Antarctic Ocean at 58°58.07' S and 06°41.98' E using ZoBell medium at an incubation temperature of 2°C (Weyland et al. 1970). The pure culture was maintained on Marine Agar 2216 (Difco, Detroit, MI, USA) at 2°C. The tests for utilization of organic compounds as sole carbon and nitrogen source were carried out in liquid basal medium seawater (BMS) (Helmke and Weyland 1984). The enzymatic activities of amylases, chitinases, ureases, and nitrate reductases were determined by the method of Weyland et al. (1970). Alginase activity was measured according to Ahrens (1968). All physiological tests were performed at an incubation temperature of 5°C.

E. coli DH5 α was used for the preparation of the gene library. *E. coli* BL21(DE3) (pLysS) was used for the overproduction of the pectate lyases. *E. coli* cells were routinely cultivated under vigorous agitation at 37°C in Luria-Bertani (LB) medium. The marine strain ANT/505 was cultivated for 2 days at 16°C on a modified ZoBell medium with 0.25% (w/v) citrus pectin (Sigma, München, Germany). The ZoBell medium with the following composition was used: 750 ml ASW [artificial seawater with a salinity of 2.7% according to Burkholder (1963)] containing 24 g/l NaCl; 5.3 g/l MgCl₂·6H₂O; 7 g/l MgSO₄; 0.7 g/l KCl; 0.01 g/l FeSO₃; 5 g/l peptone; 1 g/l yeast extract; and 10 g/l FePO₄. Before autoclaving, the pH was adjusted to 7.6.

Nucleic acid manipulation

Chromosomal DNA from strain ANT/505 was prepared according to Sambrook et al. (1989). Plasmid DNA was purified by the alkaline lysis procedure (Sambrook et al. 1989). Chromosomal DNA was partially digested with *Sau*3AI; fragments of 4 kb were isolated from 0.8% (w/v) agarose gel with the Agarose Gel DNA Extraction Kit from Roche Diagnostics (Penzburg, Germany). These fragments were ligated to *Bam*HI-digested pUC18. The ligated plasmids were transformed in competent *E. coli* DH5 α cells (Hanahan 1983) and selected on LB agar plates containing 100 mg/l ampicillin. Clones with pectinase activity were determined on LB agar plates containing 0.25% (w/v) citrus pectin after overnight cultivation at 37°C and a subsequent incubation at room temperature (RT) for 2 days. The colo-

nies of positive pectinase-producing clones were identified by staining the plates with 1% (w/v) of hexadecyl trimethyl ammonium bromide solution (Sigma) for 3 h.

The sequence of positive clones was determined by automated fluorescence sequencing with an ABI PRISM dye terminator cycle sequencing reaction mix (Perkin-Elmer, Rodgau-Jügesheim, Germany) in a 377 Perkin-Elmer DNA sequencer. The nucleotide sequences reported in this article have been submitted to the Genbank/EMBL Data Bank with the accession numbers AF278706 for *pelA* and AF278705 for *pelB*.

For the overproduction of the pectate lyases, the coding sequences of both genes were cloned into the T7 expression vector pRSET-A (Invitrogen, Groningen, Netherlands). Ligated plasmids were transformed into *E. coli* BL21 (DE3) pLysS. Construction of plasmid pRSETpelA was performed by cloning of a 1,764-bp *Bam*HI/*Eco*RI PCR fragment (primers pelAf gataaggatccgatgcaagacagcgacgtg and pelAr gcttcgaattccgcacaagagaaggaat) covering the coding region including the potential signal sequence of *pelA* into pRSET-A. Plasmid pRSETpelB was constructed in a similar way by cloning of a 2,058-bp *Bam*HI/*Eco*RI PCR fragment (primers pelBf ataaggatccgatgaaaaaac-taatatt and pelBr gcttcgaattccctaaagtctgagcgcg) covering the coding region including the potential signal sequence of *pelB* into pRSET-A.

E. coli BL21 cells carrying pRSETpelA or pRSETpelB were grown overnight on 5 ml LB. Three milliliters of the culture medium was transferred to 150 ml LB medium in a 500-ml shake flask. The culture was incubated at 37°C and 230 rpm until an optical density (OD) (600 nm) of 0.4–0.5. Subsequently, the induction was initiated by addition of 1 mM isopropyl thiogalactoside (IPTG) (final concentration). After 5 h cultivation at 30°C, the culture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant and the pellet were collected and stored at 4°C.

Purification of the pectate lyases

The cell pellet from isolate ANT/505 or from *E. coli* after overexpression was resuspended in 1/10 volume of 50 mM Tris buffer (pH 7.5), and lysozyme to a final concentration of 50 μ g/ml was added. After incubation for 30 min at 37°C, the debris of the cell wall and the protoplasts were centrifuged for 5 min at 10,000 rpm and 4°C. The periplasmic protein fraction in the supernatant was concentrated to approximately 0.5 μ g/ μ l by centrifugation (5,000 rpm, 4°C) with filter membranes (Ultrafree-30) from Millipore (Bedford, MA, USA).

The sample was applied to a DEAE Sepharose column in 10 mM Tris buffer (pH 8.5) containing 0.6 mM CaCl₂. After washing the column with the same buffer, the proteins were eluted by a gradient of 0–1 M NaCl in 200 mM Tris buffer (pH 8.5) with 0.6 mM CaCl₂. The eluted fractions showing activity on pectin acid were collected and concentrated with membrane filters as described earlier. The sample was applied to a Resource Q 6-ml column (Pharmacia Biotech, Freiburg, Germany) in 10 mM Tris

buffer (pH 8.5) containing 0.6 mM CaCl₂. After washing with the same buffer, the proteins were eluted by a gradient of 0–1 M NaCl in 200 mM Tris buffer (pH 8.5) with 0.6 mM CaCl₂. The sample was applied to a gel filtration column (Superdex 75; Pharmacia Biotech) and eluted by 10 mM Tris buffer (pH 8.5).

Enzyme assays

Citrus pectin and the potassium salt of polygalacturonic acid from oranges supplied by Sigma were used for the enzyme assays. The degree of esterification of the pectin was approximately 60%. Pectate lyase activity was measured by the determination of reducing sugars (Bernfeld 1955); 50 µl of enzyme solution was mixed with 450 µl 50 mM Tris-HCl buffer (pH 9.5) containing 0.25% (w/v) pectin acid, 20 mM NaCl, and 0.1 mM CaCl₂. The samples were incubated at 30°C for 60 min. For determination of reducing sugars, 0.5 ml of dinitrosalicylic acid solution was added. The samples were boiled for 10 min and cooled to RT. After centrifugation for 5 min at 10,000 rpm, the samples were measured spectrophotometrically at 530 nm. Sample blanks were used to correct for nonenzymatic release of the reduced sugar. One unit of pectate lyase activity was defined as 1 nM of reducing sugar liberated per minute. For the determination of unsaturated bonds according to Collmer et al. (1988), the reaction was stopped by addition of 0.5 ml sodium citrate buffer 0.4 M (pH 4.0), centrifuged at 10,000 rpm for 5 min, and measured at 235 nm in the spectrophotometer.

Protein electrophoresis and N-terminal protein sequencing

Denaturing protein gel electrophoresis was performed with a 10% (w/v) polyacrylamide gel as described by Sambrook et al. (1989). For the native protein gel electrophoresis, a 10% (w/v) polyacrylamide gel without sodium dodecyl sulfate (SDS) was used. After electrophoresis, the native gels were rinsed with Tris buffer 50 mM (pH 9.5) containing 20 mM NaCl and 0.1 mM CaCl₂ for 10 min. The native gel was overlaid with a second gel containing 0.1% (w/v) pectate in 100 mM Tris buffer (pH 9.5) with 20 mM NaCl and 0.1 mM CaCl₂ and incubated at 30°C for 1 h. The overlay gel was stained with 0.1% ruthenium red (Sigma) for 10 min and washed in distilled water. The N-terminal protein sequencing was done as described by Völker et al. (1992).

Results

Screening of cold-adapted marine bacteria with pectinolytic activity

Bacteria from water and sediment samples from the Antarctic and Arctic ocean from sea ice, seawater, and sedi-

Table 1. Differential characteristics of the species *Alteromonas haloplanktis*^a and the isolate ANT/505

Characteristics	<i>Alteromonas haloplanktis</i> ^a	ANT/505 strain
Growth at 4°C	–	+
Reduction of nitrate to nitrite	d	–
Production of:		
Amylase	d	+
Alginase	–	+
Chitinase	d	–
Urease	d	–
Utilization of:		
D-Galactose	d	+
d-Fructose	d	+
Cellobiose	–	+
Salicin	–	+
Succinate	+	–
Citrate	+	–
D-Mannitol	d	+
Glycerol	–	+
L-Arabinose	d	–
Trehalose	d	–
Acetate	+	–
Propionate	+	–
L-Serine	+	–
L-Leucine	d	–

Boldface type indicates traits useful for the preliminary identification of *Pseudoalteromonas* species

d, differs among strains

^aBergey's Manual of Systematic Bacteriology (1984)

ment were screened on ZoBell medium agar containing 0.25% (w/v) citrus pectin, apple pectin, or polygalacturonic acid. Colonies showing a clear halo after addition of ruthenium red were selected. The biggest halo showed a strain isolated from Antarctic sea ice from surface water in the Southern Ocean (Weyland et al. 1970). This isolate was called strain ANT/505 (Table 1). This strain is a psychrotolerant marine bacterium that grows in a temperature range from 0° to 29°C. The optimal growth temperature was 23°C. The 16S rDNA sequencing of isolate ANT/505 showed a 100% homology to the Antarctic strain *Pseudoalteromonas haloplanktis*.

The highest level of pectinolytic activity of isolate ANT/505 on citrus pectin or pectate was determined in the late stationary phase (Fig. 1). The major part of the pectinolytic activity could be found in the supernatant. However, the pellet also showed pectinase activity during growth on pectate or citrus pectin, indicating an additional cell wall-associated or periplasmic pectinolytic activity. This cell wall-associated pectinase activity could be released by treatment of the cells with lysozyme (data not shown). The separation of the extracellular protein fraction of a stationary ANT/505 culture in a native polyacrylamide gel overlaid with 0.1% (w/v) citrus pectin indicated that strain ANT/505

Fig. 1. Growth curve of strain ANT/505 (dotted line) cultivated at 16°C in ZoBell medium containing citrus pectin and the corresponding extracellular [on citrus pectine (solid squares) and pectate (solid circles)] or cell wall-associated [on citrus pectine (open squares) and pectate (open circles)] pectinolytic activities

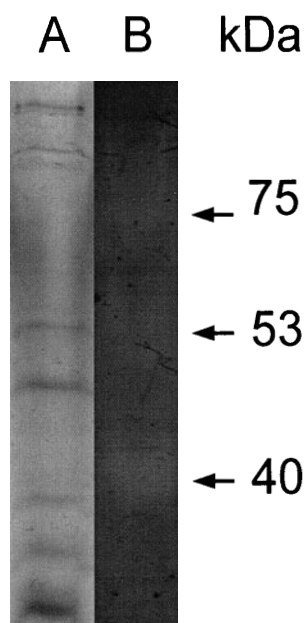
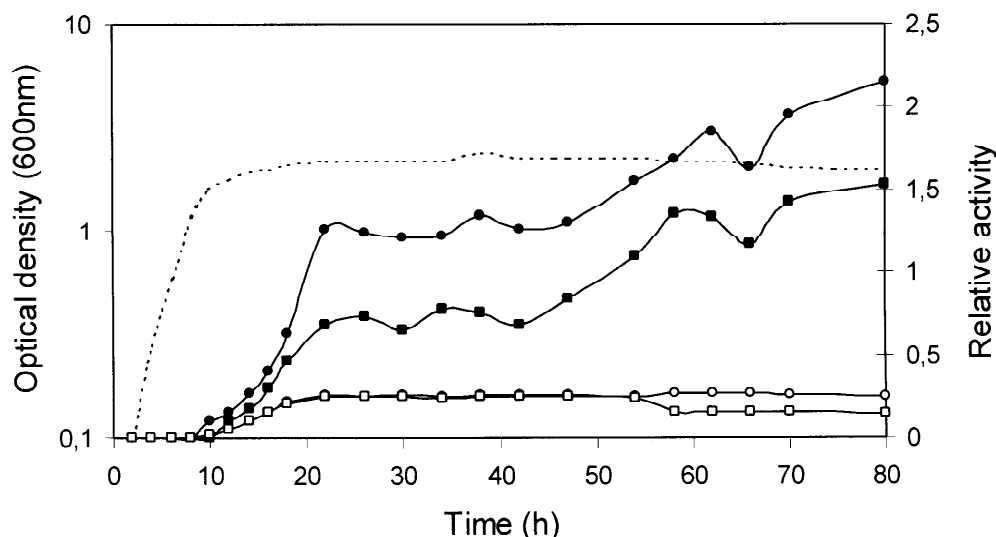


Fig. 2. Native polyacrylamide gel electrophoresis (PAGE) with extracellular protein extract of strain ANT/505 (A) stained with Coomassie blue and (B) the overlay gel with 0.1% pectin acid, stained with ruthenium red. The pectinolytic bands are indicated with arrows

secretes three different pectinases into the extracellular medium (Fig. 2).

Cloning and sequencing of two pectate lyase genes of *P. haloplanktis* strain ANT/505

The screening of approximately 15,000 clones of a genomic library of strain ANT/505 in *E. coli* DH5 α on LB-agar

plates containing 0.1% (w/v) citrus pectin showed five colonies with pectinolytic activity. The DNA inserts of the clones were 3–4 kb long. The DNA sequencing showed open reading frames (ORFs) of 1,671 and 1,968 nt, corresponding to proteins of 68 and 75 kDa, which were named PelA and PelB (Fig. 3). The N-terminal part of the deduced amino acid sequence of the two ORFs showed homology to pectate lyases from *Erwinia chrysanthemi* (Keen and Tamaki 1986; Tamaki et al. 1988) and *Aspergillus nidulans* (Ho et al. 1995). The highest homology of PelA was found to *pelA* of *A. nidulans* (48% identity). PelB showed the highest homology to *pelA* of *E. chrysanthemi* (34% identity). According to the classification of pectate lyases (Shevchik et al. 1997), PelA and PelB belong to class I. The typical conserved regions of the class I pectate lyases could be also found in PelA and PelB (Fig. 3).

PelA and PelB contain a signal peptide of 17 and 26 amino acids, respectively, with the typical features of signal peptides of the Sec-dependent secretion pathway. Both pectate lyases reveal an unusually long C-terminal part, which comprises about 190 amino acids in the case of PelA and about 300 amino acids in the case of PelB (data not shown). The C-terminal amino acid sequence of PelB showed homologies to sequences of known xylanases.

Purification and characterization of the two pectate lyases

The coding sequences of *pelA* and *pelB* were cloned into the T7 expression vector pRSET-A. For overproduction of both pectate lyases in *E. coli* (DE3) BL21, the expression system was introduced by the addition of 1 mM IPTG at 30°C followed by a further cultivation for approximately 5 h at 30°C. After overproduction of PelA or PelB in *E. coli*, only PelB could be found in the extracellular medium, whereas PelA was mainly located in the periplasm (data not shown). PelA and PelB were partially purified from the

Fig. 3. Alignment of the amino acid sequences of the pectate lyases PelA and PelB of strain ANT/505 with known pectate lyases of class I. The C-terminal amino acid sequence of PelA and PelB is not shown. Conserved regions are indicated by boxed areas. The grade of conservation of the appropriate amino acids is indicated by an asterisk (highly conserved), a colon (medium conserved), or a dot (low grade of conservation)

	10	20	30	40	50	60
E. chrys. pelB	-----MKSLITPIAAGLLLAFSQYSLAAD-----	TGGYTKTDGGDVSQVAVKKA				
E. chrys. pelC	-----MKSFIAPIAAGLLLAFSQSSLAA-----	TGCYATTSGGNVTVGAUSKTA				
E. chrys. pelD	-----MNNTRVSSVGTKS--LLAAIATSMTWTSVNAATLQPT--	KATEAASTG-WATQGGGTTG				
E. chrys. pelE	-----MKNTVRVSIGTKS--LLAAVVTAALMATSAAYAA-----	VETDAATTG-WATQGGGTTG				
E. chrys. pelA	-----MMNKASGRSFTFRSSKYLATLIAGMMASGVSAEELVSD--	KALESAPTQVWASQNGFTTG				
A. nidul. pelA	-----MRFPTPLFLAAVAIASPAPDLNARHELTR--	RQASESCPIGYCTQNGGTTG				
ANT/505 pelB	-----MKKTN--ILKYSLHTTALAIAIYGG--	SALANASGYASTNGNTTG				
ANT/505 pelA	-----MQDSDVVNLLERNNAIFASFNDGQGWTPPEPLAIPILPDEPIAITPVNNSLGFAGYNFSLTG					
Prim.cons.	MMNNTVRVSMK32S53LAAGL23A2MASSLAAA4L4T4PI22T2AATTGGWATQNGGTTG					
	70	80	90	100	110	120
E. chrys. pelB	S3MQDIVNIEAAKVDANGKVKV--GGAYPLVITYTG	NEDS-LINAAAANICGG	W3KDKAR			
E. chrys. pelC	ASMQDIIDIIDAAKVDSDGKVKV--GGAYPLVITYTG	NEDS-LINAAAANICGG	W3KDKAR			
E. chrys. pelD	GAKAASAKIYAVKNISEFKALNGTDDPKFIQVTC	AIDI--SGGKAYTSFDDQ--KARSQI				
E. chrys. pelE	GAKAA--KAVEVKNISDFPKKALNGTSSAKI	IKVTCFIDI--SGGKAYTSFDDQ--KARSQI				
E. chrys. pelA	GAAATSNDNIYIVTNISEFTSALS--AGAEAKI	IQIKGCFIDI--SGGTPYDFADQ--KARSQI				
A. nidul. pelA	GAAGD---TVTVTNLADLLEAAE--SDGPL	IIIVSGSIS--G-----S-AKI				
ANT/505 pelB	GAGGDVVYATTGTQIHQALCNRA--SSDPT	IIIVVEGFIINHNTSKVSGDSCNTGDPDLEL				
ANT/505 pelA	GEGGTVVVDNMGTLKSLAQAQ--SLGVPE	IIIVVDCIITDANSG--GDNSS----IEI				
Prim.cons.	GA42D3V2I32VTNIS3F4KALKG3GAYP2IIQVTC2IDI2SGGKAY2DFCDQGR2RSQI					
	130	140	150	160	170	180
E. chrys. pelB	GVEIKDFTKGLTIIGANGSSANFGLI	WVNVN--SDIIVVNRNRIS	-----YLPGG--A			
E. chrys. pelC	GVEIKDFTKGLTIIGANGSSANFGLI	WVNVN--SDIIVVNRNRIS	-----YLPGG--A			
E. chrys. pelD	SVPSTNTIIG---IGSNGKFTNGSLV	IKG---VENVLLRNLYIETPV	DVAAPHYESGDGWN			
E. chrys. pelE	SIPSTNTIIG---VGSNGKFTNGSLV	IKG---VKNVLLRNLYIETPV	DVAAPHYESGDGWN			
E. chrys. pelA	NIPANTTVIG---LGTDAKFINGLI	IDGDTGNNVIRNRYIETP	IDVEPHYERKGDGWN			
A. nidul. pelA	RVASDKTIFG---ESGSSITGIF	YIR---VENVLMNRNKIS	-----KVAD--N			
ANT/505 pelB	KEISNVSIIG---VSGALFDQLGI	HIRS--SENIIIQNVHVRN	-----VKKSGSPIS			
ANT/505 pelA	KMDNVSIIG---VADRGELSGI	AIAR---ANNIIIQNLKI	-----EVLTTG--K			
Prim.cons.	3VPSNTTIIIG2TI2GSNG2FTNGGL2IRGTDG2SNVIRNLYI2TPVDVAAPHY22GDGWN					
	190	200	210	220	230	240
E. chrys. pelB	QDGMFRIDN--SPN	WLDHNEIFAANHECDG--TKDGD--TT	FESADIKKGGATYVTIS			
E. chrys. pelC	QDGMFRIDN--SPN	WLDHNEIFAANHECDG--TKDGD--TT	FESAPDIKGGATYVTIS			
E. chrys. pelD	AEWDAVIDN--STR	WVDHVTISDGSFTDDKYTTKDG	EKYVQHDGALDIKKGSDYVTIS			
E. chrys. pelE	AEWDAVIDN--STN	WVDHVTISDGSFTDDKYTTKDG	EKYVQHDGALDIKKGSDYVTIS			
E. chrys. pelA	AEWDAAMNITNG--AHH	WIDHVTISDGNFTDDMYTTKDG	EYVQHDGALDIKKGSDYVTIS			
A. nidul. pelA	--GDAIGIDA--SSN	WVDHCDLSDGLSCGKD-----	LDGLVDISHGAEWITVS			
ANT/505 pelB	NGGDAIGMESN--VRN	WVDHVTLEASGGESSG-----	YDALDMKNNTKYVTLIS			
ANT/505 pelA	DGISIEGDENKPTAN	WIDHNEIYSSLNVDDQ-----	YDGLIDSKSGAENITIS			
Prim.cons.	AEGDA3GIDN3PS2NWWVDHVTLS2GNF2DDGYTTKDGKEYVQHDGALDIKKGATYVTIS					
	250	260	270	280	290	300
E. chrys. pelB	YNYTHGVKVKGLSGFSSSD	FAERN---ITYHNNIYSDV	NARLFLQ	RGGNVHAYNNLYTG		
E. chrys. pelC	YNNIHGVKVKGLAGFSSSD	BAERN---ITYHNNIYNDV	NARLFLQ	RGGNVHAYNNLYTN		
E. chrys. pelD	SSRFELHDKTILICHSDSN	SSDQSGKLVTFHNNV	FDRVTERTPR	VRFGSIHAYNNVYL		
E. chrys. pelE	YSRFELHDKTILIGHSDSN	SSDQSGKLVTFHNNV	FDRVTEREAP	VRFGSIHAYNNVYL		
E. chrys. pelA	NSLIDQDKTMLIGHNDTN	BAQDKGLVLEFNNV	FNVRTERA	PRVYRGSIHGFNNVFKG		
A. nidul. pelA	NTYFHDHWKGLIGHSDN	DEDLGHLVTVANNY	YVYVSRTP	PLIRFATVHIINNYDS		
ANT/505 pelB	YSILRNSGRGGLVGSSDSD	DANGP---VTFHNNY	QNNRSRTP	PLLRHATAHAYNNYSG		
ANT/505 pelA	YNYIHDWKTSLHGSDD	SSNKNR--HITFHHNR	FENI	ISRVLPREGOGHIFNNY		
Prim.cons.	YSYI1H3DKTGLIGHSDS2SA2D3GKLVHVT2H2NVF22V22RTPLVRFSG2HAYNN2Y2G					
	310	320	330	340	350	360
E. chrys. pelB	ITSSGLNVRQNGKAL	IENNWFENAVSPVTSRYDGSNFG--TWVLKGN	NITKPADFATYN-			
E. chrys. pelC	ITSSGLNVRQNGKAL	IENNWFENAVNVTSRDYSNFG--TWVLKNN	NITKPADFATYN-			
E. chrys. pelD	DVKHNSVYPYLYSFLGTS	---GSILSESNSTLSNLSIDGKN	PECSIVKQPNKVFSD			
E. chrys. pelE	DVKHNSVYPYLYSFLGTS	---GSILSESNSTLSNLSIDGKN	PECSIVKQPNKVFSD			
E. chrys. pelA	DAKDPVYRYQYSPGIGTS	---GSLVSEGNSTIANLS---	ASKACKVVKPFNGSIFSD			
A. nidul. pelA	LIDTGVNCRMDAQVLIQS	---SAFHNC	PDRAIFPADS---	VYTGAVVDDVLDGSS-		
ANT/505 pelB	IQSSGMNPRIGGKIRAEN	---NYFQDSK	DPGLGTFYTN----	DMYVQVSG---	NINDN	
ANT/505 pelA	ITSSAINSRMGAELHIEN	---NYFEHT	KNPVVSFYSK----	VIGYWNTS	GNYLGE	GVTW
Prim.cons.	ITSSGVNPRQY22GLIE2NWFEN271SF3N2FT2SNLKI22V2K3CN1VK2F2324FSD					
	370	380	390	400	410	420
E. chrys. pelB	ITWTADTKAYRNADTWT	STGTYPYYSYSPVSAQC	VKDKLANYAGV	GKNL	LATLASSACK	
E. chrys. pelC	ITWTADTKAYRNADTWT	STGTYPYYSYSPVSAQC	VKDKLASVAGV	GKNL	LAELTSAACK	
E. chrys. pelD	NGSLVNGSSTTKLDT	CGLTAYKPTLPYKYS---	AQTM	TSSLASSINS	NAGYGL-----	
E. chrys. pelE	KGSLVNGSSTTKLDT	CGLTAYKPTLPYKYS---	AQTM	TSSLATS	INNAGYGL-----	
E. chrys. pelA	NGSVLNGS--AVD	LSGCGFSAYTSKIPYIYD--	VQPM	TEAQSITD	NAGSGLK-----	
A. nidul. pelA	--NSVPEGT-----	LTPSSLPYAAIT---	ALGSG	OVASVI	PCTAGOKL-----	
ANT/505 pelB	IDWSEDESKLHPAGNP	SPSSTT	SISIPYNYQ	LDNTQ	CVPAI	TAATAGANKL
ANT/505 pelA	GDVADGDVAEVTAT	GMP	TSSYQAP	YKLT	TPVMDVKA	VIHAGIC
Prim.cons.	IGS322GSA3222DTCGSTAT3PTLPY2Y82VSAQC2V282LAASAGV2KGLGL494ACK					

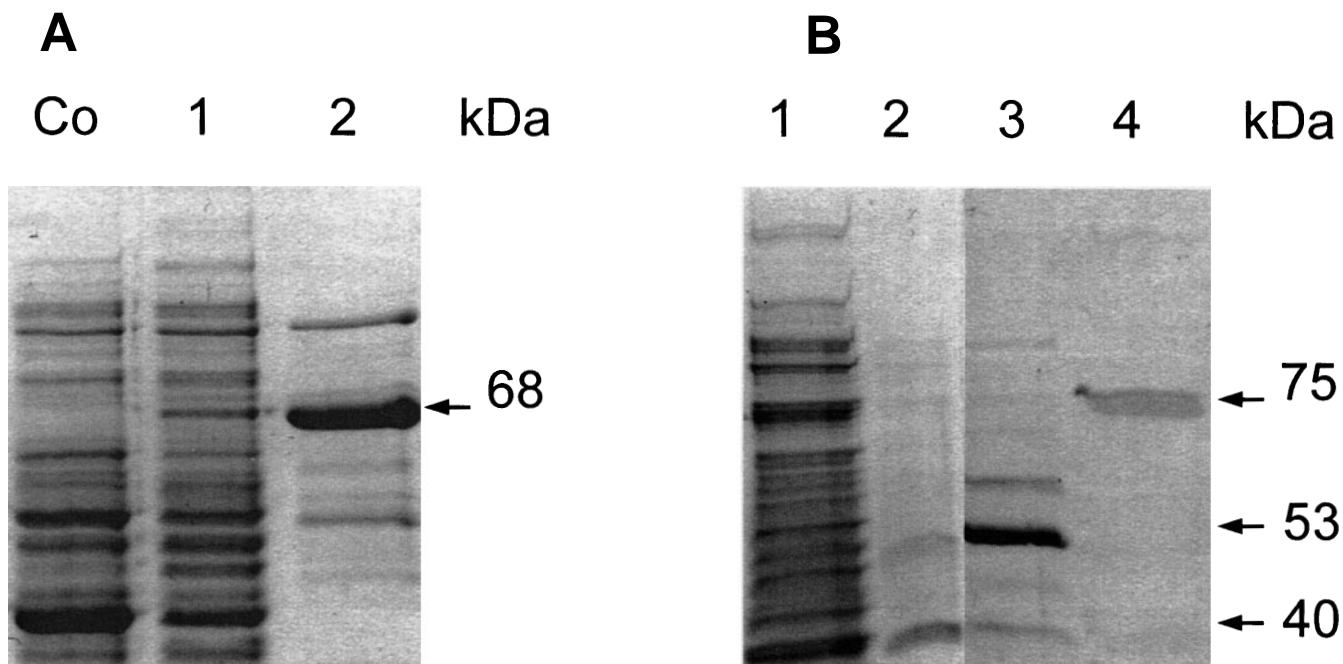


Fig. 4A, B. SDS-PAGE of the pectate lyases of strain ANT/505 after overproduction with the T7 expression system in *E. coli* BL21 and after purification. **A** PelA: Co, *E. coli* (DE3) BL21 control without overexpression; 1, periplasmatic protein fraction of *E. coli* BL21 cells after overproduction of PelA; 2, purified PelA protein fraction. **B** PelB: 1, extracellular plus periplasmatic protein fraction of *E. coli* BL21 cells after overproduction of PelB; 2, first purified PelB protein fraction; 3, second purified PelB protein fraction; 4, third purified PelB protein fraction

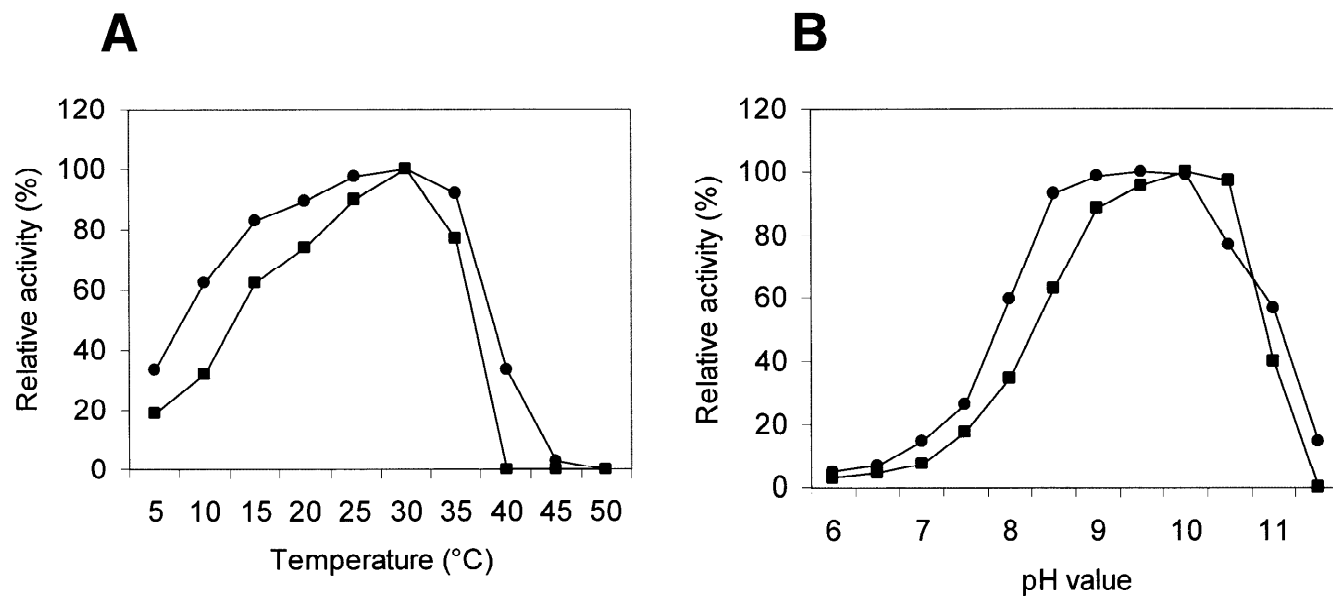


Fig. 5A, B. Effect of temperature (**A**) and pH (**B**) on the activity of the purified pectate lyase PelA (circles) and PelB (squares)

periplasmatic and extracellular *E. coli* protein fraction by ion-exchange chromatography. The purified pectate lyase protein fractions of PelA and PelB were determined by N-terminal sequencing of the first six amino acids. For PelA, an N-terminal amino acid sequence of SFNDGQ could be determined. In the case of PelB, three different fractions with pectinolytic activities

were isolated (Fig. 4). The size of the biggest fragment of 75 kDa is consistent with the theoretical molecular weight of 75 kDa from PelB deduced from its amino acid sequence. The N-terminal sequencing of these fragments showed AIYGGs in all cases, which is consistent with the theoretically deduced amino acid sequence of PelB. These N-terminal amino acid

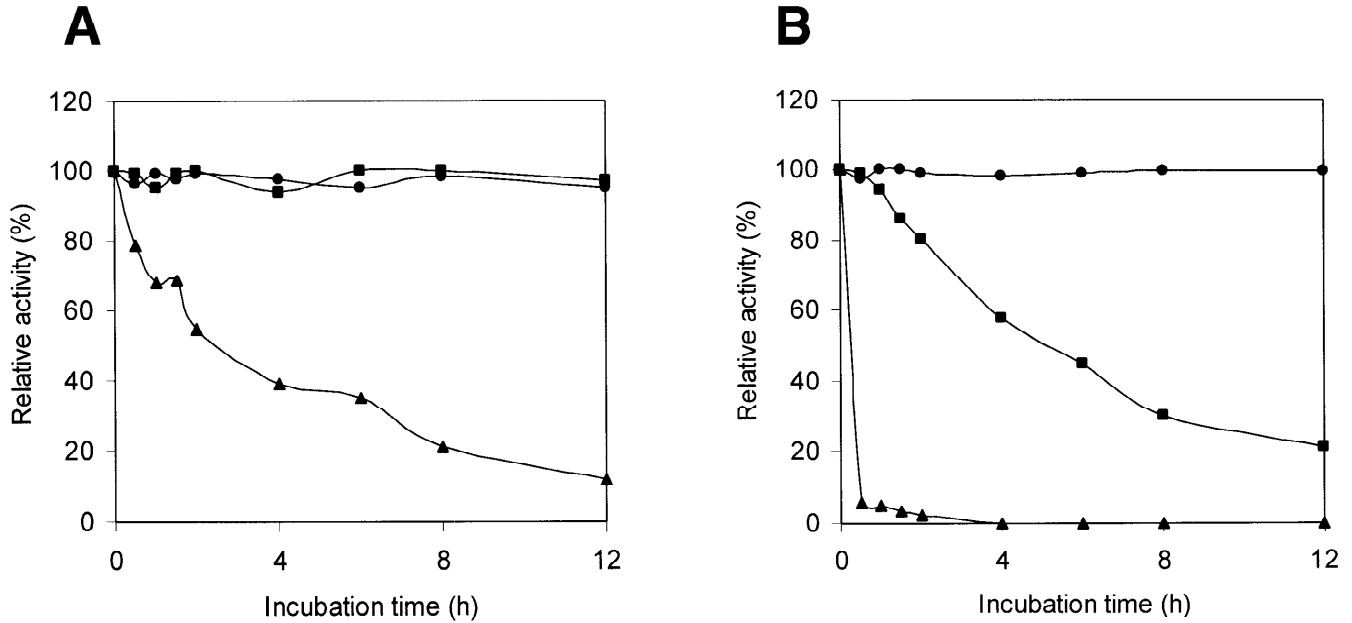


Fig. 6A, B. Thermostability of the pectate lyases PelA (A) and PelB (B) at 40°C (triangles), 30°C (squares), and 20°C (circles)

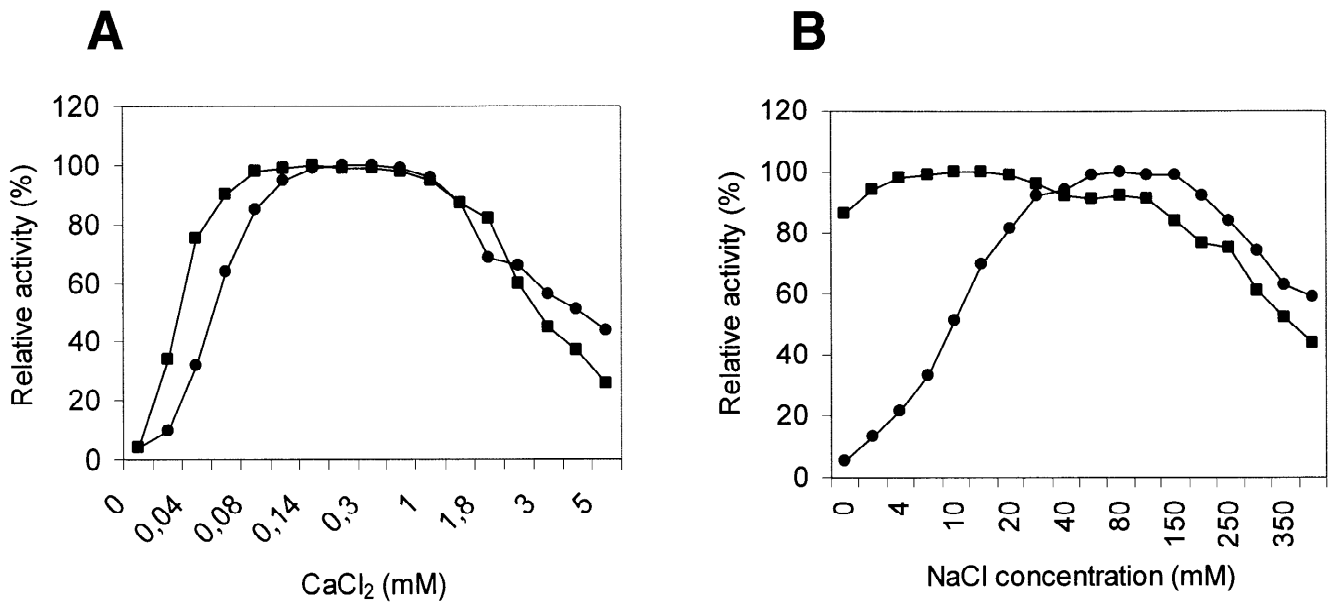


Fig. 7A, B. Dependence of the enzymatic activities of PelA (circles) and PelB (squares) on the presence of (A) Ca²⁺ and (B) Na⁺

sequences revealed that the signal peptides of both pectate lyases were correctly processed in *E. coli*.

For enzyme characterization, the purified protein fractions were used. The maximal enzymatic activities of both pectate lyases were found at 30°C (Fig. 5A). The remaining pectate lyase activity at 5°C was 25% for PelA and 35% for PelB. The optimal enzyme activity of PelA and PelB could be found at a pH range of 9–10 (Fig. 5B), which is a typical

pH of pectate lyases (Whitaker 1991). Both enzymes revealed a higher enzymatic activity with pectate than with pectin (data not shown). The K_m values of both lyases for pectate and citrus pectin were 1 and 5 g/l, respectively. PelA showed a higher stability at 30°C than PelB (Fig. 6). Although PelA did not show a decrease in enzymatic activity at 30°C during a 12-h period (Fig. 6A), the half-life of PelB at 30°C was approximately 4 h (Fig. 6B).

A typical feature of pectate lyases is the dependence of their enzymatic activity on the presence of Ca^{2+} (Whitaker 1991). PelA and PelB required 0.14–1 M Ca^{2+} for optimal activity on pectic substrates (Fig. 7 A), whereas the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) resulted in complete inhibition of the enzymes (data not shown). In contrast to PelB, the pectate lyase activity of PelA was also dependent on Na^+ (Fig. 7B); no enzyme activity of PelA could be measured if NaCl was not added to the medium. The presence or absence of KCl did not influence the enzyme activities of PelA and PelB.

Discussion

Little is known about pectin-degrading marine microorganisms. To date, pectinolytic marine microorganism have not been described in the literature. The strain ANT/505 investigated in this study is thus the first pectinolytic marine bacterium isolated from a permanently cold environment such as the Antarctic Ocean. According to the 16S rDNA sequencing, strain ANT/505 belongs to *P. haloplanktis*. Despite the 100% homology of the 16S rRNA of this isolate to *P. haloplanktis*, the isolate ANT/505 differs in selected catabolic activities such as succinate, citrate, or glycerol utilization. Similar differences in catabolic activities could be found between two subspecies of *P. haloplanktis* (Gauthier et al. 1995).

The sequencing of the five pectinolytic clones of an ANT/505 gene library in *E. coli* and a sequence alignment with known protein sequences indicated that both pectinases belong to the group of pectate lyases (EC 4.2.2.2). This classification of the cloned pectinases of strain ANT/505 was supported by the enzyme characterization, which showed the two typical features of pectate lyases, dependence of enzymatic activities on an alkaline pH and the presence of Ca^{2+} (Whitaker 1991). The activity of the pectate lyase PelA of strain ANT/505 was dependent on sodium; other monovalent cations such as K^+ could not replace Na^+ . A similar dependence on sodium has been found with the pectate lyase PelE from *E. chrysanthemi* (Tardy et al. 1997). Furthermore, the determination of the formation of unsaturated bonds indicated that the enzymatic reaction of PelA and PelB of strain ANT/505 is based on the cleavage of the glycosidic bonds by a β -elimination (data not shown), which is the typical reaction of lyases (Collmer et al. 1988).

Several pectate lyases from mesophilic bacteria, above all from members of the genus *Erwinia* but also from the gram-positive genus *Bacillus*, have been cloned and characterized (Pissavin et al. 1996; Tardy et al. 1997; Shevchik et al. 1997; Bauer and Collmer 1997; Soriano et al. 2000). Recently, a thermostable pectate lyase from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus*, has been isolated and characterized (Kozianowski et al. 1997). Laurent et al. (2000) identified three cold-adapted pectate lyases from *Chryseomonas luteola* MFCL0. These enzymes macerated celeriac stored at low temperature.

However, to our knowledge no pectate lyase from a cold-adapted microorganism has been cloned so far.

There is a significant homology of PelA and PelB of strain ANT/505 to the pectate lyases PelA, PelB, PelC, PelD, and PelE of *E. chrysanthemi* (Condemine and Robert-Baudouy 1991) and to PelA of *A. nidulans* (Ho et al. 1995). According to the classification of known pectate lyases (Shevchik et al. 1997), PelA and PelB could be affiliated to class I of this type of pectinases.

The overproduction of PelA or PelB in *E. coli* BL21 (DE3) with the exceptionally strong T7 expression system surprisingly did not result in a strong synthesis of these enzymes. Tierny et al. (1999) observed that recombinant vectors harboring a functional gene for a pectate lyase were rapidly lost in *E. coli* during the absence of selective pressure. They suggested that this plasmid instability was caused by to a toxic effect of the *pel* gene product when overproduced and was closely related to both a decrease of the growth rate and the impossibility of transforming different strains of *E. coli* with the recombinant plasmids harboring a functional *pel* gene. When the expression level of the *pel* gene was reduced, the stability was greatly improved. Such a toxic effect of pectate lyases could be at least one explanation for the low level of PelA and PelB from strain ANT/505 after the overexpression in *E. coli*.

Furthermore, the highest pectate lyase activity of PelB during the overexpression in *E. coli* could be observed when the optical density measured at 600 nm at the time of induction was not higher than 0.5 and optimal aeration of the culture was ensured. A higher cell density during induction with IPTG gave very low PelB activity in *E. coli* BL21 shake-flask cultures. It has been shown that the expression and activity of PelC, a pectate lyase from *E. carotovora*, was dependent on DsbA in *E. coli* (Humphreys et al. 1995). This periplasmic protein of *E. coli* is involved in the formation of disulfide bonds of secreted enzymes. Also, in the major *E. chrysanthemi* pectate lyases it has been shown that the cysteine residues are involved in disulfide bond formation (Yoder et al. 1993). In the case of PelB from strain ANT/505, two cysteines could be found. Thus, oxidative conditions could be required for the formation of disulfide bonds of the pectate lyase PelB and could thus determine the activity of this enzyme during the overexpression in *E. coli* BL21.

The thermoflexibility of cold-adapted enzymes is supposed to be prerequisite for the high catalytic activity of these proteins at low temperature (Gerday et al. 2000). However, this adaptation of psychrophilic proteins to low-temperature conditions also determines an instability of these proteins at higher temperatures. Feller et al. (1998) observed that the cold-adapted amylase of *P. haloplanktis* cannot be functionally expressed in *E. coli* at 37°C. Significant amylase activity could be only found at temperatures below 25°C. During overexpression of the pectate lyases PelA and PelB of strain ANT/505 in *E. coli*, the highest enzyme activity could be determined in both cases at 30°C (data not shown). At 37°C, a significantly lower activity for both pectate lyases could be observed. As PelB showed a half-life of approximately 5 h at 30°C, it can be concluded

that a lower temperature (e.g., 25°C) would give a higher pectate lyase activity. However, because the T7 expression system works optimally in a temperature range from 30° to 37°C, a compromise between the thermostability of such cold-adapted enzymes and the optimal temperature for the expression system must be found.

The native polyacrylamide gel of the extracellular protein fraction indicated that the isolate ANT/505 has, ultimately, three extracellular pectinases. However, the overproduction of PelB in *E. coli* showed the same pectinase pattern in the supernatant, indicating that the three different extracellular pectinolytic activities in the wild-type strain ANT/505 are the result of degradation or processing of PelB. This assumption is supported by the N-terminal sequencing, whereby it could be demonstrated that the PelB fragments purified from the *E. coli* supernatant have the same correct N-terminal sequence.

Both enzymes, PelA and PelB, reveal an unusually long C-terminal part, which does not show any homology to other known pectate lyases. The C-terminus of PelA revealed a weak homology to the fimbrial adhesion protein of *E. coli* (Schmoll et al. 1989) and to the flagellin of *Salmonella typhimurium* (Homma et al. 1990). It is possible that this C-terminal domain plays a role in the localization of PelA in the cell wall compartment of *P. haloplanktis* ANT/505 and *E. coli*. The C-terminal part of PelB showed homologies to both xylanases of *Streptomyces thermoviolaceus* (Tsujiro et al. 1997) and to *Streptomyces coelicolor* (Redenbach et al. 1996). Because the smaller processed PelB fragments missing the C-terminus showed a pectinolytic activity similar to that of the larger wild-type fragment, this part of the enzyme is obviously not involved in the PelB pectate lyase activity. The processing of PelB remains obscure. For the pectate lyase PelI of *E. chrysanthemi*, processing at the N-terminus by extracellular host proteases has been described (Shevchik et al. 1998). It is interesting to note that the proteolytic processing of PelI also did not alter the pectate lyase activity. Thus, similar to the processed C-terminal part of PelB from strain ANT/505, the 97 N-terminal amino acids of PelI also are not essential for enzymatic capacity.

The marine Antarctic bacterium *P. haloplanktis* ANT/505 was isolated from more than 500 strains from the Antarctic and Arctic Oceans as the only bacterium that produced significant pectinolytic activities under the conditions used in this study. This finding reflects the negligible role of pectin as a carbon source in these permanently cold marine environments. The two pectate lyases described in this study represent the first pectinases found in marine microorganisms.

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