# Characterization of the pectinolytic enzymes of the marine psychrophilic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505

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# Chapter I

# 1. Introduction

## 1.1. Psychrophylic bacteria

The earth is dominanted by low-temperature environments. Over 80% of the earth's biosphere is cold, and about 90% of the oceans is colder than 5°C (Gounot 1999). Many microorganisms of the marine environments are adapted to these low temperatures and classified as psychrophiles or psychrotolerant organisms.

The first mention of the term 'psychrophile' was apparently made by Schmidt-Nielsen in 1902 (Morita 1975) for the description of bacteria capable of growing at 0°C. There are in fact no formal reasons to restrict the term psychrophile to bacteria, or to prokaryotes. Various species of yeast, algae (Hohman 1975), (Loppes, Devos et al. 1996), insect (Lee and Denlinger 1991), fish (Eastman 1993) and probably plant can be referred to as psychrophiles if they continuously experience low temperatures, for example below an arbitrary limit of 5°C. Among microorganisms, which can grow over a temperature span of 20°C or more, it is necessary to distinguish psychrophiles from psychrotolerant organims because of differences in the ecological distribution and biochemical adaptations of both groups (Russell 1990), (Russell 1992). The widely accepted definition by Morita proposes that psychrophiles are microorganisms, which are able to grow at temperatures below 0°C, and which have an optimal growth temperature below 15°C. Psychrophiles do not grow at 20°C and above. Psychrotolerants grow better at temperatures above 20-25°C and may have upper limit as high as 40°C (Morita 1975).

The classical definition of Morita is frequently used in the literature. However, this definition is ambiguous for three main reasons. First, the temperature limits have been arbitrarily selected and do not correspond to any clear separation of biological processes or environmental conditions. Second, Morita's definition does not apply to most eukaryotes. Finally, microorganisms behave as thermodynamic units: increasing the culture temperature increases reaction rates and the growth rate (Feller and Gerday 2003). For that reasons, recently several authors have used the general term psychrophiles to designate all microorganisms growing well at temperatures around the freezing point of water instead of psychrophilic and "psychrotrophic" microorganisms terms (D'Amico, Collins et al. 2006), (Russell 1998).

## 1.2. Habitats of psychrophilic bacteria

Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and polar regions. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or the ice, and to brine channels. These contain high concentrations of salts, exopolymeric substances and/or particulate matter, and fluid flow is maintained by concentration and temperature gradients. Despite all of these challenges, life thrives in these environments with a remarkable microbial biodiversity of mainly bacteria, fungi (in particular yeasts) and microalgae. Among the bacteria that have been detected, the most commonly reported microorganisms are the Gram-negative α-, β- and γ-proteobacteria (*Pseudomonas* spp. and *Vibrio* spp.) and the *Cytophaga–Flavobacterium–Bacteriodes* phylum. Coryneforms, *Arthrobacter* sp. and *Micrococcus* sp. are the most frequently found Gram-positive bacteria (Nichols, Nichols et al. 1995). The Archaea appear to be poorly represented omongst psychrophilic populations. They are most readily were isolated from naturally cold environments and that are most amenable to laboratory cultivation are methanogens. Isolates have come from an Antarctic lake (Franzmann, Liu et al. 1997), (Franzmann, Stringer et al. 1992), a freshwater lake in Switzerland (Simankova, Parshina et al. 2001), in cold marine sediment in Alaska (Chong, Liu et al. 2002) and in the Baltic Sea (Singh, Kendall et al. 2005), (von Klein, Arab et al. 2002).

For growth at low temperatures psychrophilic microorganisms must possess enzymes, which have high activity under these conditions. These enzymes are called cold-adapted enzymes.

## 1.3. Cold-adapted enzymes

### 1.3.1. What are cold-adapted enzymes?

Cold-adapted enzymes are enzymes which have high activity at low temperatures. Typically, the specific activity of cold-adapted enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0-30°C. At higher temperatures, denaturation of the cold enzyme occurs (Gerday, Aittaleb et al. 2000). Jones at el. reported about a fructose-1,6-bisphosphate aldolase and a gluco-6-phosphate dehydrogenase from *Vibrio marinus,* which lost 100% of activity within 30 min at 35°C and 36°C (Jones, Morita et al.

1979). Morita reported that a partially purified malic dehydrogenase from *Vibrio marinus* MP-1, which has a temperature optimum between 15-20°C, was inactivated at temperatures above 20°C (Morita 1975). Furthermore, a lactate dehydrogenase of a psychrophilic *V. marinus*, which has a maximal activity at 10-15°C, showed no activity at 40°C (Mitchell and et 1985).

Ohgiya was the fist person who has suggested that the enzymes isolated from cold-adapted microorganisms can be classified into three groups (Fig 1.):

Group I: Heat-sensitive. The characteristics of these enzymes are similar to mesophilic enzymes.

Group II: Heat-sensitive and relatively more active than mesophilic enzymes at low temperature.

Group III: A thermostability as mesophilic enzymes but more active than mesophilic enzymes at low temperature.

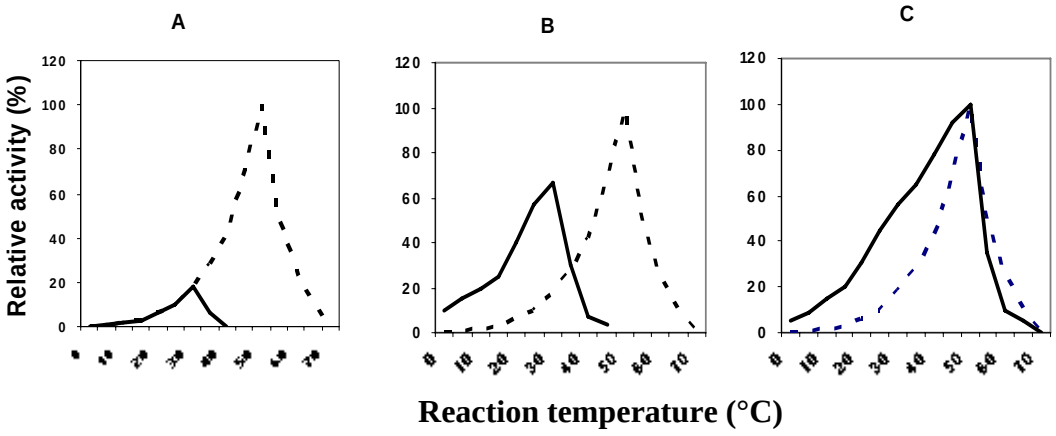



**Fig. 1.** Three types of enzymes isolated from cold-adapted microorganisms. (A) Group I, (B) Group II, (C) Group III. Dotted line: typical thermoprophiles of enzymes isolated from mesophiles or thermophiles. Solid lines: typical thermoprophiles of enzymes isolated from cold-adapted microorganisms. Adapted from Ohgiya et al. (1999).

### 1.3.2. Enzyme catalysis at low temperatures

According to Raymond (Raymond, Wilson et al. 1989), any decrease in temperature causes an exponential decrease of the reaction rate, the magnitude of which depends of $\Delta G^*$ ($\Delta G^*$ is the activation free energy). Accordingly, most biological systems, including the single biochemical reaction, display a reaction rate 2 to 3 times lower when the temperature is decreased by 10 °C (Q10 = 2 to 3). As a consequence, the activity of a mesophilic enzyme is between 16 and 80 times lower when the reaction temperature is shifted from 37°C to 0°C.

Thus, it is surprising that metabolic rates of Antarctic fish are only slightly lower than those of temperate water species and that the generation time of psychrophilic bacteria near 0°C are of the same order as those of mesophilic microorganisms at. Clearly, psychrophilic organisms have found mechanisms of temperature compensation in order to cope with the reduction of chemical reaction rates inherent to low temperatures (Feller and Gerday 1997).

### 1.3.3. Low stability of cold-adapted enzymes

The stability of cold-adapted enzymes relates the structural factors, which is responsible for stability of the three dimensional structure of enzyme. It seems that all the structural factors that stabilize a protein molecule can be attenuated in both strength and number in psychrophilic enzymes (Russell 2000; Smalas, Leiros et al. 2000). The number of proline and arginine residues (which restrict backbone rotations and can form multiple hydrogen bonds and salt bridges, respectively) is reduced, whereas clusters of glycine residues (which essentially have no side chain) provide localized chain mobility. All weak interactions (ion pairs, aromatic interactions, hydrogen bonds and helix dipoles) are less abundant, and non-polar core clusters have a weaker hydrophobicity, making the protein interior less compact. Frequently, stabilizing cofactors bind weakly, and loose or relaxed protein extremities seem to favour unzipping (Feller and Gerday 2003). In multimeric enzymes, the cohesion between monomers is also reduced by decreasing the number and strength of interactions that are involved in association (Bell, Russell et al. 2002). However, each protein family adopts its own strategy to decrease stability by using one or a combination of these structural alterations.

### 1.3.4. The active site of cold-adapted enzymes

Structural prediction by homology modeling has recently reached sufficient level of reliability to allow close inspection of the psychrophilic enzyme conformation. The active site of psychrophilic enzymes were noted that all amino acid residues involved in the reaction mechanism, as well as all side chains pointing towards the catalytic cavity, are strictly conserved with respect to their mesophilic homologues. This has been shown by the perfect conservation of ~30 side chains that are involved in the binding of a transition-state analogue in both psychrophilic and mesophilic α-amylases (glycosidases that hydrolyse starch) (Aghajari, Feller et al. 1998). Holland et al. have suggested that structural adaptations outside the active site are thought to modify the dynamic properties of the catalytic residues, leading

to cold activity (Holland, McFall-Ngai et al. 1997). Aghajari et al. have observed no point mutations occur in the catalytic center of *A. haloplanctis* α-amylase indicating a hypothesis of mutations affecting the flexibilty should be preferred in cold-adapted enzymes.

Recently, Tsuruta et al.(2005) have been reported a crystal structure of cold-active protein-tyrosine phosphatase (CAPTPase) of a psychrophile, *Shewanella* sp. The catalytic residue of CAPTPase is histidine, as opposed to the cysteine of known protein-tyrosine phosphatases (PTPases). They were speculated that the hydrophobic moiety around the catalytic residue of CAPTPase might play an important role in eliciting high activity at low temperature (Tsuruta, Mikami et al. 2005)

### 1.3.5. Structural determinants of cold-adapted enzymes

The structural basis of the adaptation of enzymes to low temperature conditions is still not completely understood. The fist crystal structure of a cold-adapted enzyme was generated from the α-amylase of the psychrophilic bacterium *Alteromonas hulopfuncris* (called AHA) (Aghajari et al. 1998). The three-dimensional structure of AHA resembles those of other known alpha-amylases of various origins with a surprisingly greatest similarity to mammalian alpha-amylases (called MAA) (Aghajari, Feller et al. 1998). In comparison to the fife disulfide bridges of MAA only four disulfide bridges are found in the psychrophilic α-amylase. Thus, AHA has one less disulfide bridges compared to MAA. This seems to be an important difference as the extra disulfide bridge in the mesophilic enzyme MAA connects two of its protein domains and presumably causes thus limited movements of these domains. This difference gives the psychrophilic enzyme a larger degree of overall structural flexibility (Aghajari et al. 1998). Another feature of this cold-adapted enzyme is less ion bounds. Thus, is for example the calcium ion binding affinity, which is conserved in all known α-amylases, in AHA $10^4$ times lower than compared to the mesophilic α-amylases from porcine pancreas (Feller, Payan et al. 1994). It was suggested that the potentially higher flexibility of the heat-labile AHA is also determined by the weaker calcium ion interactions.

Two other types of molecular adaptation of cold-adapted proteins have been shown by X-ray structures for a cold-active citrate synthase from an Antarctic bacterium (Russell, Gerike et al. 1998) and two crystal forms of the alkaline protease from an Antarctic *Pseudomonas* specie (Aghajari, Van Petegem et al. 2003). It was found that the catalytic cavity seems to be larger and more accessible to ligands in psychrophilic enzymes than in mesophilic enzymes. This improved accessibility is thought not only to be responsible for the accommodation of

the substrate at low energy cost, but also to facilitate the release and exit of the reaction products (Feller and Gerday 2003).

### 1.3.6. Cold-acclimation proteins and Antifreeze proteins

Cold-acclimation proteins (CAPs) seem to be another important and general feature of cold-adapted microorganisms (Hébraud, Potier et al. 2000). This set of ~20 proteins is permanently synthesized during steady-state growth at low temperatures, but not at mild temperatures (Hebraud, Dubois et al. 1994; Berger, Morellet et al. 1996). Some of the few CAPs that have been identified in cold-adapted bacteria are cold-shock proteins in mesophiles, such as the RNA chaperone CspA (Berger, Normand et al. 1997). It has been proposed that these CAPs are essential for the maintenance of both growth and the cell cycle at low temperatures, but their function is still poorly understood.

Antifreeze proteins have been widely studied in polar fish (Jia, DeLuca et al. 1996). These peptides and glycopeptides of various sizes decrease the freezing point of cellular water by binding to ice crystals during formation, thereby inhibiting their growth. Although antifreeze proteins have been reported in several eukaryotes, there is no supporting evidence for the occurrence of such glycopeptides in psychrophilic bacteria.

## 1.4. The industrial application of cold –adapted enzymes

Most industrial enzymes are produced by microorganisms. Almost all bacterial enzymes for the industry are produced by mesophiles. Thermophilic enzymes usually star in discussion of industrial uses because their heat-stability makes them ideal biocatalysts for many reactions. However, in some cases (example as food and daily industry), enzymatic reactions have to be carried out at low temperature. In such cases, the application of cold-adapted enzymes could be more useful than enzymes from mesophiles. The biological application of cold-adapted enzymes has a great potential, because:

- **Energy saving.** Cold- active enzymes can held to save energy
- **Saving of labile or volatile compounds.** In biotransformations of food-processing volatile or labile compounds can be saved by application of cold-adapted enzymes at low temperatures.
- **Prevention of contamination.** Food-processing at low temperatures prevents the growth of mesophilic contaminants.

- **Inactivation of enzymes.** These thermoflexible enzymes can be easily inactivated moderate temperatures

| Applications | Enzymes | Advantages |
|---|---|---|
| *Detergent* | Protease, lipase, cellulase, etc. | Use in tap water |
| *Food industries* | | |
| - Modification of constituents | β-Galactosidase, lipase | Keeping freshness |
| - Improvement of taste and flavour | Protease, lipase, etc. | Keeping freshness |
| - Removal of fish skin | Protease | Maintaining product quality |
| - Clarification of fruit juice | Pectinase, cellulase | Keeping fragrance |
| - Preservation | Lysozyme, glucose oxidase | Improved preservative effect |
| *Enzymatic synthesis* | Lipase, nitrile hydratase | For volatile and heat sensitive materials |
| *Treatment of waste water* | Catalase | Less energy consumption |
| *Biotechnology* | | |
| - Protoplast formation | Cell wall digesting enzymes | High viability |
| - Molecular biology | Phosphatase, uracil DNA glycosylase | Complete heat intactivation |

**Table 1**. Industrial applications of enzymes at low temperatures (Ohgiya 1999).

Some typical applications of cold-adapted enzymes are summarised in Table 1. The enzymes used in detergents are proteases, lipases, α-amylases and cellulases. The advantages are reduction in energy consumption and a reduction in wear and tear in the cold washing. The drawback of using cold-adapted enzymes is their instability when added to the final product during long time storage. However, these enzymes are usually recombinant enzymes and because of the increasing knowledge of the structural background and improved techniques for site-directed but also random mutagenesis it might be possible to increase the stability of cold-adapted enzymes (Narinx and et 1997; Taguchi, Ozaki et al. 1998). In fabric production of tissues often cotton fibres ends protruding from the main fibres, which reduce the smoothness and alter the appearance of the garment (Gerday, Aittaleb et al. 2000). The application of cold-adapted cellulase could increase the smoothness and the softness of tissues by removal of these over-hanging cotton fibres and would allow a rapid inactivation of the enzymes after the treatment. Cold-adapted β-galactosidases could be used to reduce the amounts of lactose in milk. The applications of pectinases in the fruit juice industry have improved the juice extraction process and reduce the viscosity in products. The application of cold-adapted pectinases would allow a fruit juice processing at low temperatures. These

would save the quality of the fruit juice and enables an inactivation of the enzyme at moderate temperatures after treatment.

### 1.5. Degradation of pectin by pectinases

#### 1.5.1. Structure of plant cell wall

Higher plants contain a number of polysaccharides. These include starch, cellulose, hemicelluloses, pectic substances and other polysaccharides. The pulp of fruits and vegetables is mainly composed of cells. The cells were surrounded by a cell wall (Roland 1980). This is what separates and maintains the integrity of the cell in the pulp and give them their rigidity. The plant cell walls can be divided into three layers: middle lamella, primary wall and secondary wall, (Keegstra and el. 1973; Monro, Penny et al. 1976; Dey and Brinson 1984). The primary wall is mainly composed of protopectin. The middle lamella is mainly composed of soluble pectin connecting one cell to another. This structure floats in a watery gel formed of different hemicellulose fraction, e.g. xylans, xyloglucans, arabinogalactans (Carpita and Gibeaut 1993). Different groups of polysaccharides can be found in cell walls of higher plants shown in Table 2. Pectins are linked through proteins to xyloglucans and cellulose matrix by hydrogen bonds, or covalent bonds (Mac Cann and Roberts 1994).

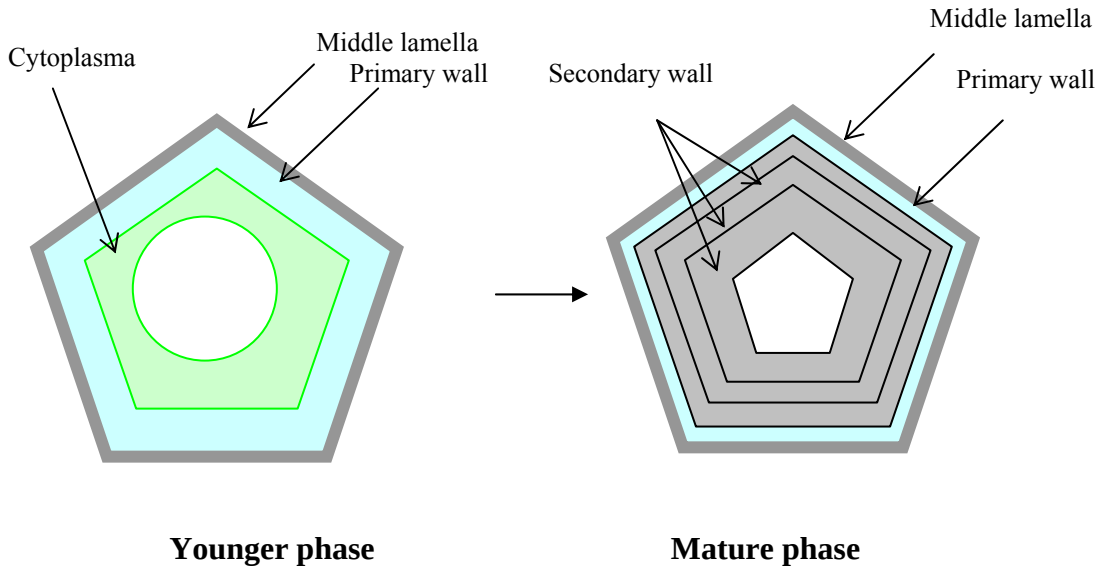



**Fig 2**. Structure of the plant cell wall. Adapted from Alberts et al. (Alberts, Bray et al. 1986)

| General category | Structural classification |
|---|---|
| Cellulose | β-D-Glucan (4-linked) |
| Pectic substances | Galacturonans and rhamnogalacturonans |
| | Arabinans |
| | Galactans and arabiogalactans I* |
| Hemicelluloses | Xylans (including arabinoxylans and (4-O-methy) glucuronoxylans) |
| | Galactomannans and glucomannans |
| | β-D-Glucan (3-and 4- linked) |
| | β-D-Glucan-callose (3-linked) |
| | xyloglucans (4-linked β-D-glucans with attached side chains) |
| Other polysaccharides | Arabinogalactans II* |
| | Glucoromannans |

**Table 2.** Polysaccharides of cell walls in higher plants. According to Aspinall (1969, 1980). *Arabinogalactans of type I are essentially linear and contain 4-linked β–D-galactan chains, whereas those of type II contain branched 3- and 6-linked β-galactan chains. The polysacharides may occur in part as proteoglycans or polysaccharide-protein conjugates.

### 1.5.2. Structure of pectin

The pectic substances consist of a number of compounds: protopectin, pectin (polymethylgalacturonate), pectin acid (polygalacturonate) and oligogalacturonates. The parent compound in the immature tissue is protopectin. The protopectin is an insoluble substance. Pectin is a soluble polymeric material and at least 75% of the carboxyl groups of the galactoronate units are esterified with methanol. Pectin acid is a soluble polymeric material and less than 75% galacturonate units are methylated. Oligogalacturonates are smaller polymers of about two or more galacturonate units. Oligomethylgalacturonates are polymers with two or more galacturonate units, which are partially or completely methylated on C-6 (Whitaker 1990).

Pectin substances represent a group of closely related polysaccharides (Godfrey and West 1996). Two fundamental constituents of pectic substances are galacturonans and rhamnogalacturonans in which the C-6 carbon of galactose is oxidized to a carboxyl group, the arabinans, the galactans and arabinogalactans I (Table 2). The rhamnogalacturonan is the

major constituent of the pectic substances. The primary chains consist primarily of α-D-galacturonate units linked (1-4) with 2-4% of L-rhamnose units linked β-(1-2) and β-(1-4) to the D-galacturonate units. The side chains of rhamnogalacturonans are of various compositions and lengths. The extended side chains are usually homogeneous polymers of either D-galacturonic acid units or of L-arabinose units (Whitaker 1990).

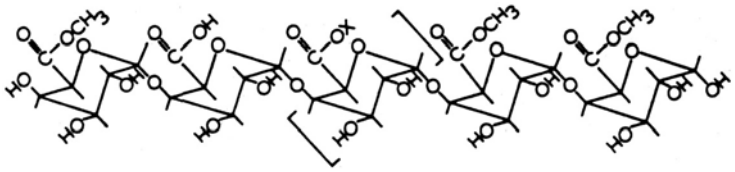


**Fig 3.** Chemical structure of a pectin segment.

The general chemical structure of pectins is shown in Fig. 3. The D-galacturonate units are linked together by α-1-4 glycosidic bonds. Some of D-galacturonate residues are modified by methyl-esterification at the carboxyl groups at O-6 or acetyl- esterification on the hydroxyl groups at O-2 or O-3 position (de Vries and Visser 2001), others are in $COO^-$ or –COOH forms depending on the pH. The degrees of methylation and acetylation vary greatly, depending on the source of pectin (Schols, Posthumus et al. 1990)

### 1.5.3. Pectinases

#### 1.5.3.1. Classification of pectinases

The pectolitic enzymes are generally classified on the basis of their action toward the galacturonan part of the pectin molecules. The pectolytic enzymes include two esterases, six polygalacturonases and four lyases (Table 3).

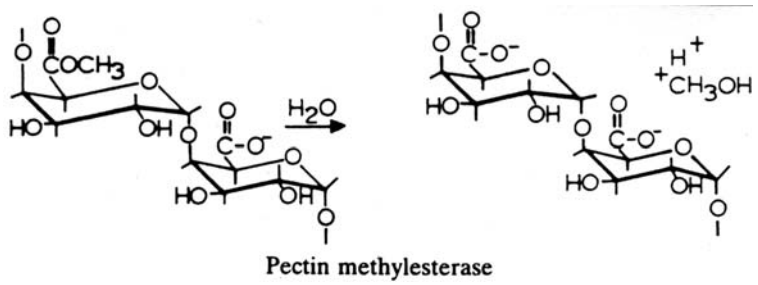
| Name | EC No. | Primary substrate | Products | Mechanism |
|---|---|---|---|---|
| **Esterase** | | | | |
| - Pectin methylesterase ( pectin esterase) | 3.1.1.11 | Pectin | Pectin acid + Methanol | Hydrolysis |
| - Pectin acetylesterase* | | Pectin | | |
| **Polygalacturonase** | | | | |
| - Protopectinase | 3.2.1.15 | Protopectin | Pectin | Hydrolysis |
| - Endopolygalacturonases | 3.2.1.82 | Pectin acid | Oligogalacturonates | Hydrolysis |
| -Exopolygalacturonases | | Pectin acid | Monogalacturonate | Hydrolysis |
| - Oligogalacturonate hydrolases | | Trigalactoronate | Monogalacturonate | Hydrolysis |
| - 4:5 unsaturated oligo-galacturonate hydrolase | | 4:5 (galacturo-nate)$_n$ | Unsaturated monogala-turonate and saturate (n-1) | Hydrolysis |
| - Endopolymethylgalactu-ronases | | Pectin | Methyloligogalacturo-nates | Hydrolysis |
| **Lyase** | | | | |
| - Endopolygalacturonate lyases (endopectate lyase) | 4.2.2.2 | Pectin acid | Unsaturated oligogalac-turonates | *Trans* elimination |
| - Exopolygalacturonate lyase (exopectate lyase) | 4.2.2.9 | Pectin acid | Unsaturated digalactu-ronates | *Trans* elimination |
| - Oligogalacturonate lyase | 4.2.2.6 | Unsaturated digalacturonate | Unsaturated monoga-lacturonates | *Trans* elimination |
| - Endopolymethylgalactu-ronate lyase (endopetin lyases) | 4.2.2.10 | Pectin | Unsaturated methyloli-gogalacturonates | *Trans* elimination |

**Table 3.** Classification of pectinolytic enzymes accoding to (Whitaker 1990).
* A new pectinolytic enzyme found recently.

### 1.5.3.2. The degradation of pectic substrates by pectinases

There are two basic reaction mechanisms of pectinases: hydrolysis and *trans* elimination (Table 3). The hydrolysis mechanisms required the presence of water. In contrast to hydrolysis, *trans* elimination mechanisms react in the absence of water and give a product with a double bond (Whitaker, 1990).

**Pectin methylesterase** (EC 3.1.1.11) acts upon pectin to remove methoxyl groups from the C-6 carboxyl groups of galacturonate units by hydrolysis (Fig. 4). The final product of the reaction is pectin acid, methanol and $H^+$ from ionisation of carboxyl groups. The release of $H^+$ from the newly formed carboxyl groups will decrease the pH of the reaction media. Thus, the pectin methylesterase activity can be determined by the measurement of the pH of the solution after the reaction (Whitaker 1990).



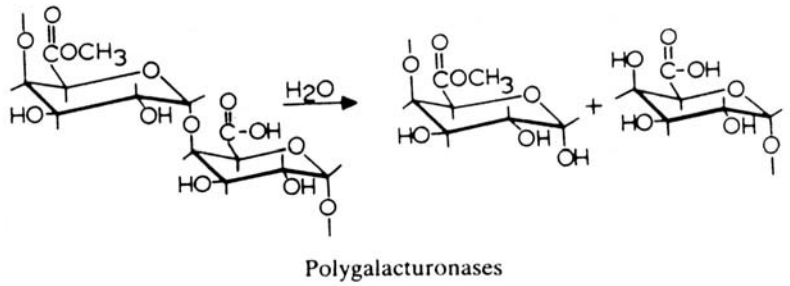

$$\text{Pectin} + n\text{H}_2\text{O} \xrightarrow{\text{methylesterases}} \text{pectin acid} + \text{methanol}$$

**Fig. 4.** The hydrolysis of pectin by pectin methylesterases.

**Pectin acetylesterase** hydrolysis pectin by removing acetyl groups from C2 and C3 hydroxyl groups of galacturonate units. (Searle-van Leeuwen, Vincken et al. 1996) detected the first pectin acetylesterase in *Aspergillus niger*. (Shevchik and Hugouvieux-Cotte-Pattat 1997) found two further pectin acetylesterase in *Erwinia chrysanthemi* 3937. However, the studies of pectin acetylesterase are so far limited.

**Endopolygalacturonase** (EC 3.2.1.15) and **exopolygalacturonase** (EC 3.2.1.67) hydrolyses internal glycosidic bonds of polygalacturonates (Fig. 5). The products of the reaction are a series of intermediate size polygalacturonates (for endopolygalacturonase) or galacturonate (for exopolygalacturonase). The activity of both enzymes can be measured by the

determination of the rate of formation of reducing groups with 3,5-dinitrosalicylate (Bernfeld 1955).




Polygalacturonases

endopolygalacturonase

Polygalacturonates + $nH_2O$ ----------→ oligogalacturonates + galacturonate

exopolygalacturonase

$(Polygalacturonate)_n$ + $nH_2O$ ----------→ $(polygalactoronate)_{n-1}$ + galacturonate

**Fig. 5**. Polygalacturonate hydrolysis by endo- and exopolygalacturonases.

**Endopolymethylgalacturonase** hydrolyses polymethylgalacturonates randomly to oligomethylgalacturonates (Albersheim and Kilias 1962; Finkelman and Zajic 1978). The reaction catalyzed by this class of enzymes is shown below:

endopolymethylgalacturonase

$(Polymethylgalacturonate)_n$ +$nH_2O$ ----------→ oligomethylgalacturonates

The activity of endopolymethylgalacturonase can also be measured by determination of the rate of reducing groups formed by the hydrolysis of the glycosidic bonds.

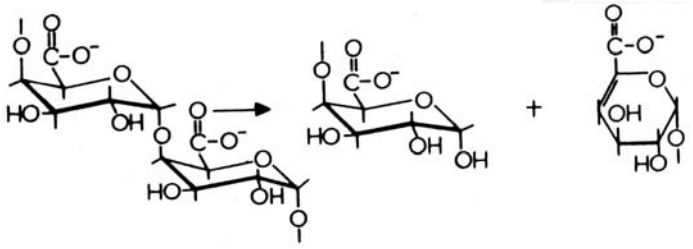
**Endopolygalacturonate lyases** (EC 4.2.2.2), which are found only in microorganisms, differ from endogalacturonases. They have pH optima in the range of 8-10, much higher than for other pectinolitic enzymes. They have an absolute requirement for $Ca^{2+}$ and they split the glycosidic bond via a *trans* elimination to give double bonds between C-4 and C-5 of the galacturonate (Whitaker 1990). The best method for the determination of the enzymatic activity is analysis of double bonds in the spectrophotometer at 235 nm (Macmilan and Phanff

1966). The activity can also be measured by the reducing sugar method (Somogyi 1952; Bernfeld 1955). The reaction catalysed by endopolygalacturonate lyases is as follows:

endopolygalacturonate lyase

Polygalacturonate ----------→ Δ 4:5 unsaturated oligogalacturonates

**Exopolygalacturonate lyases** (EC 4.2.2.9) have been found only in a few bacteria [*Clostridium multifermentans,* (Macmillian and et 1964; Macmillian and et 1966); *Erwinia aroideae* (Okamoto 1963; Okamoto 1964); *Erwinia disolvens* (Castelien and et 1976). The preferred substrate is polygalacturonate but not polymethylgalacturonate. The products are generally unsaturated Δ4:5 digalacturonates. The pH optimum of these enzymes is between 8.0 - 9.5. The activity of exopolygalacturonate lyases can be assayed as described for the endopolygalacturonate lyases. The enzymatic reaction of endopolygalacturonate lyases is shown in Fig 6.




exopolygalacturonate lyase

Polygalacturonate ----------→ Δ 4:5 unsaturated galacturonates

**Fig. 6.** Degradation of polygalacturonate by exopolygalacturonate lyases.

**Endomethylgalacturonate lyases** (EC 4.2.2.10) degrade pectin in a random fashion to the end product tetramethylgalacturonate. The reaction catalysed by endopolymethylgalacturonate lyases is as follow:

endomethylpolygalacturonate lyase

Polymethylgalacturonate ----------→ unsaturated methyloligogalacturonates

The activity of these enzymes can also be measured by the reducing sugar method (Somogyi 1952; Bernfeld 1955) or by the determination of double bonds at 235 nm in a spectrophotometer (Macmillian and et 1966).

### 1.6. The industrial application of pectinases

#### 1.6.1 Application of pectinases in the fruit juice industry

Fruits can be processed to produce juice or concentrates. The addition of pectinases to the fruits helps the juice extraction process. The addition of pectinases to the fruit mash (about 40-200 g enzyme/ton) for 30-60 min at 15-30°C decreases the viscosity by pectin hydrolysis and thus increases the yield (Godfrey and West 1996). Pectinases are also added to the juice after pressing for depectinization. Pectinase hydrolyses the residual pectin and hemicelluloses. This reaction allows the concentration of the sugar concentration in the juice up to 70-72°Brix, this also increases the storage capacity and avoids microbial contamination (Godfrey and West 1996).

#### 1.6.2. Application of pectinases in the textile industry

Mature cotton fiber is composed of a thin primary wall and a thick secondary cell wall. The goal of cotton processing is removal of the primary cell wall. The chemical composition of the fiber is ~95% cellulose and ~5% non-cellulosics. The majority of the non-cellulosic compounds are found in the primary cell wall, which is a complex lattice of pectin (partially methoxylated polygalacturonic acid), protein, cellulose, hemicellulose, and waxes. The secondary cell wall consists almost entirely of cellulose (Batra 1985; Seagull, Oliveri et al. 2000). Pectinolytic enzymes could target specifically the pectin from cotton fiber could reduce or eliminate the use of harsh chemicals, lessening the environmental burden while maintaining the integrity and strength of the cotton fiber.

#### 1.6.3. Application of pectinases in the Biotechnology

The plant cell wall Cold-adapted pectinases and cellulases are even suitable for protoplast preparation in plant biotechnology (Ohgiya 1999).

## 2. Scope of the thesis

The marine phytoplancton at permanent cold environments produces a variety of structural polymers such as cellulose and xylan. Although relatively little data are available about the hydrolysis of such compounds under low temperature conditions, it is obvious that these structural polymers can be degraded by microorganisms. Furthermore, pectin degrading microorganisms have not been reported before the start of this PhD thesis. Although pectin is of secondary importance for the marine environment, it was an aim of this thesis to investigate whether there are marine, especially cold-adapted microorganisms, which are able to degrade pectin. Such pectinolytic microorganisms should be taxonomically classified. Potential pectinolytic genes should be cloned and characterized.

# Chapter II

# Cloning of two pectate lyase genes from the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505 and characterization of the enzymes

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Keywords: Pectate lyase, pectin, psychrophilic, marine, *Pseudoalteromonas haloplanktis*

## Abstract

A marine Antarctic, psychrotrophic 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 taxonomical affiliation to *Pseudoalteromonas haloplanktis*. The supernatant of this strain showed after growth on citrus pectin three different pectinolytic activities. 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 of 1671 and 1968 nt corresponding to proteins of 68 and 75 kDa. 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 peptide 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 a temperature of 30 °C and a pH range of 9-10. The Km values of both lyases for pectate and citrus pectin were 1 $g \cdot l^{-1}$ and 5 $g \cdot l^{-1}$, respectively. Calcium was required for activity on pectic substrates, while 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.

## Introduction

The marine phytoplancton at permanent cold environments produces a variety of structural polymers such as cellulose and xylan. Although relatively little data are available about 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 the screening for xylan and cellulose degrading microorganisms from the 3 °C cold marine sediment of shallow water 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 1990). 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 fare restricted to psychrotrophic spoilage bacteria such as different strains of *Pseudomonas fluorescens* (Schlemmer, Ware et al. 1987). Recently, the production of pectate lyases by the psychrotrophic bacterium *Chryseomonas luteola* has been described (Laurent, Buchon et al. 2000). This bacterium, which has been isolated from spoiled celeriac, is able to macerate plant tissue at low temperatures due to at least three extracellular pectate lyases.

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

## Material 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 ZoeBell medium at an incubation temperature of 2 °C (Weyland, Rüger et al. 1970). The pure culture was maintained on Marine Agar 2216 (Difco) at 2 °C. The tests for utilization of organic compounds as sole

carbon and nitrogen source were carried out in liquid basal medium BMS (Helmke and Weyland 1984). Amylase, chitinase, urease, and nitrate reductase were determined by the method of Weyland et al. (1970) and alginase according to the method of Ahrens (Ahrens 1968). All physiological tests were performed at an incubation temperature of 5 °C.

E. coli DH5α was used for the preparation of the gene libraries. 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 $^{o}$C in Luria-Bertani (LB) medium. The marine strain ANT/505 was cultivated 2 days at 16 °C on a modified ZoeBell medium with 0.25 % (w/v) citrus pectin (Sigma). The ZoBell medium with the following composition was used: 750 ml ASW (Artificial Sea Water with a salinity of 27 $^{0}/_{00}$ according to Burkholder (Burkholder 1963) containing 24 g/l NaCl; 5.3 g/l $MgCl_2 \cdot 6H_2O$; 7 g/l $MgSO_4$; 0.7 g/l KCl; 0.01 g/l $FeSO_3$; 5 g/l peptone; 1 g/l yeast extract and 10 g/l $FePO_4$. Before autoclaving, the pH was adjusted to 7.6.

**Nucleic acid manipulation**

Chromosomal DNA from strain ANT/505 was prepared according to Sambrock et al. (1989). Plasmid DNA was purified by the alkaline lysis procedure (Sambrook, Fritsch 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. These fragments were ligated to *Bam*HI-digested pUC18. The ligated plasmids were transformed in competent *E. coli* DH5α 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 following incubation at RT for 2 days. The colonies 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) in a 377 Perkin Elmer DNA sequencer. The nucleotide sequences reported in this paper 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). Ligated plasmids were transformed into

*E. coli* BL21 (DE3) pLysS. Construction of plasmid pRSETpelA was performed by cloning of a 1764 bp *Bam*HI/*Eco*RI PCR-fragment (primers pelAf gataaggatccgatgcaagacagcgacgtg and pelAr gcttcgaattccgcacaaagagaaaggaat) 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 2058 bp *Bam*HI/*Eco*RI PCR-fragment (primers pelBf ataaggatccgatgaaaaaaactaatatt and pelBr gcttcgaattccttaaagttctgagcgcg) 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 ml 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 OD(600nm) of 0.4-0.5. Subsequently the induction was initiated by addition of 1 mM IPTG (final concentration). After 5 h cultivation at 30 °C the culture was centrifuged at 10000 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 the overexpression were resuspensed in 1/10 volume of 50 mM Tris buffer (pH 7.5) and lysozyme with a final concentration of 50 µg/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 10000 rpm and 4 °C. The periplasmatic protein fraction in the supernatant was concentrated to approximately 0.5 µg/µl by centrifugation (5000 rpm, 4 °C) with filter membranes (30,000 MWCO) from Millipore.
The sample was applied to DEAE Sepharose column in 10 mM Tris buffer (pH 8.5) containing 0.6 mM $CaCl_2$. After washing of 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 $CaCl_2$. The eluted fractions showing activity on pectin acid were collected and concentrated with membrane filters as described above. The sample was applied to a Resource Q 6 ml column (Pharmacia Biotech) in 10mM Tris buffer (pH 8.5) containing 0.6 mM $CaCl_2$. After washing with the same buffer, the proteins were eluted by a gradient of 0 – 1 M NaCl in 200mM Tris buffer (pH 8.5) with 0.6 mM $CaCl_2$. 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 orange supplied by SIGMA was 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). 50µl 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 $CaCl_2$. The samples were incubated at 30 °C for 60 min. For the determination of reducing sugars, 0.5 ml of dinitrosalycilic acid solution was added. The samples were boiled for 10 min and cooled down to RT. After centrifugation for 5 min at 10000 rpm, the samples were measured spectrophotometrically at a wave length of 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 Na-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

The denaturating 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 SDS was used. After the electrophoresis the native gels were rinsed with Tris buffer 50mM (pH 9.5) containing 20 mM NaCl and 0.1 mM $CaCl_2$ 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 $CaCl_2$ 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. (Volker, Mach 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, sea water and sediment were screened on ZoeBell 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, Rüger et al. 1970). This isolate was called strain ANT/505 (Tab. 1). This strain is a psychrotrophic marine bacterium,

which grows in a temperature range from 0 – 29°C. The optimal growth temperature was 23 °C. The 16S rRNA sequencing of isolate ANT/505 showed a 100 % homology to the Antarctic strain *Pseudoalteromonas haloplanktis*.

**Table 1.** Differential characteristics of the species A*lteromonas haloplanktis** and the isolate ANT/505.

| Characteristics | *Alteromonas haloplanktis** | ANT/505 strain |
|---|---|---|
| **Growth at 4°C** | **-** | **+** |
| Reduct. 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 | - |

*Bergey´s Manual of Systematic Bacteriology (1984)
d, differs among strains; Boldface type indicates traits useful for the preliminary identification of *Pseudoalteromonas* species

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 polyacrylamid gel overlaid with

0.1 % (w/v) citrus pectin indicated that strain ANT/505 secrets three different pectinases into the extracellular medium (Fig. 2).

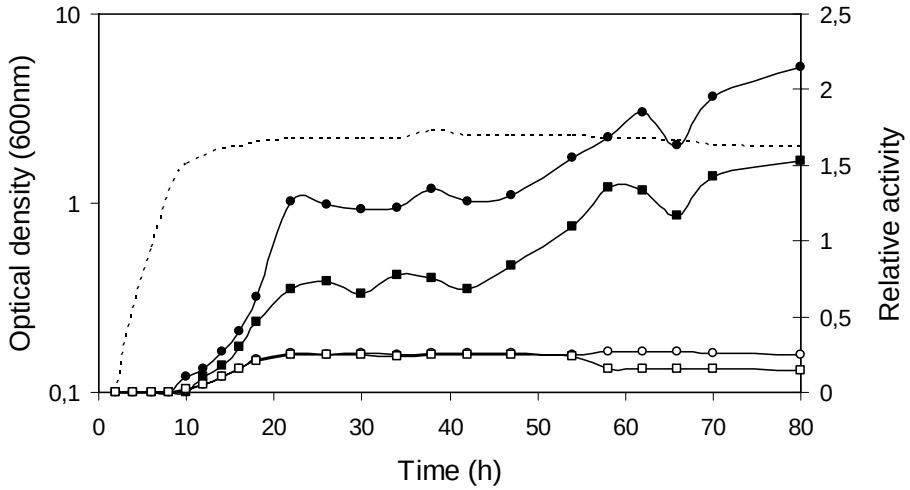


**Fig. 1** Growth curve of strain ANT/505 (dotted line) cultivated at 16°C in ZoeBell medium containing citrus pectin and the corresponding extracellular (on citrus pectine (-■-) and pectate (-●-) or cell wall associated (on citrus pectine (-□-) and pectate (-○-) pectinolytic activities.

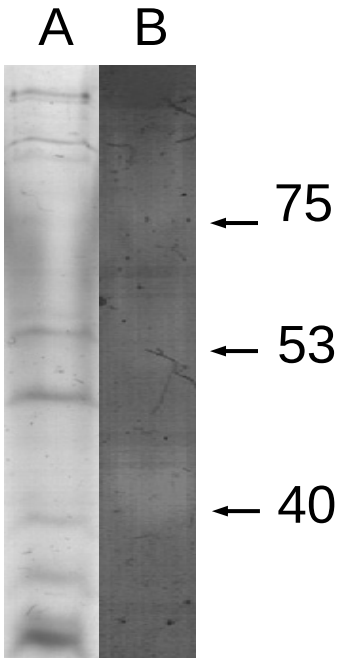


**Fig. 2.** Native PAGE with extracellular protein extract of strain ANT/505 (**A)** stained with Coomassie blue and (**B**) the overlay gel with 0.1 % pectin acid and stained with Ruthenium red. The pectinolytic bands are indicated with arrows.

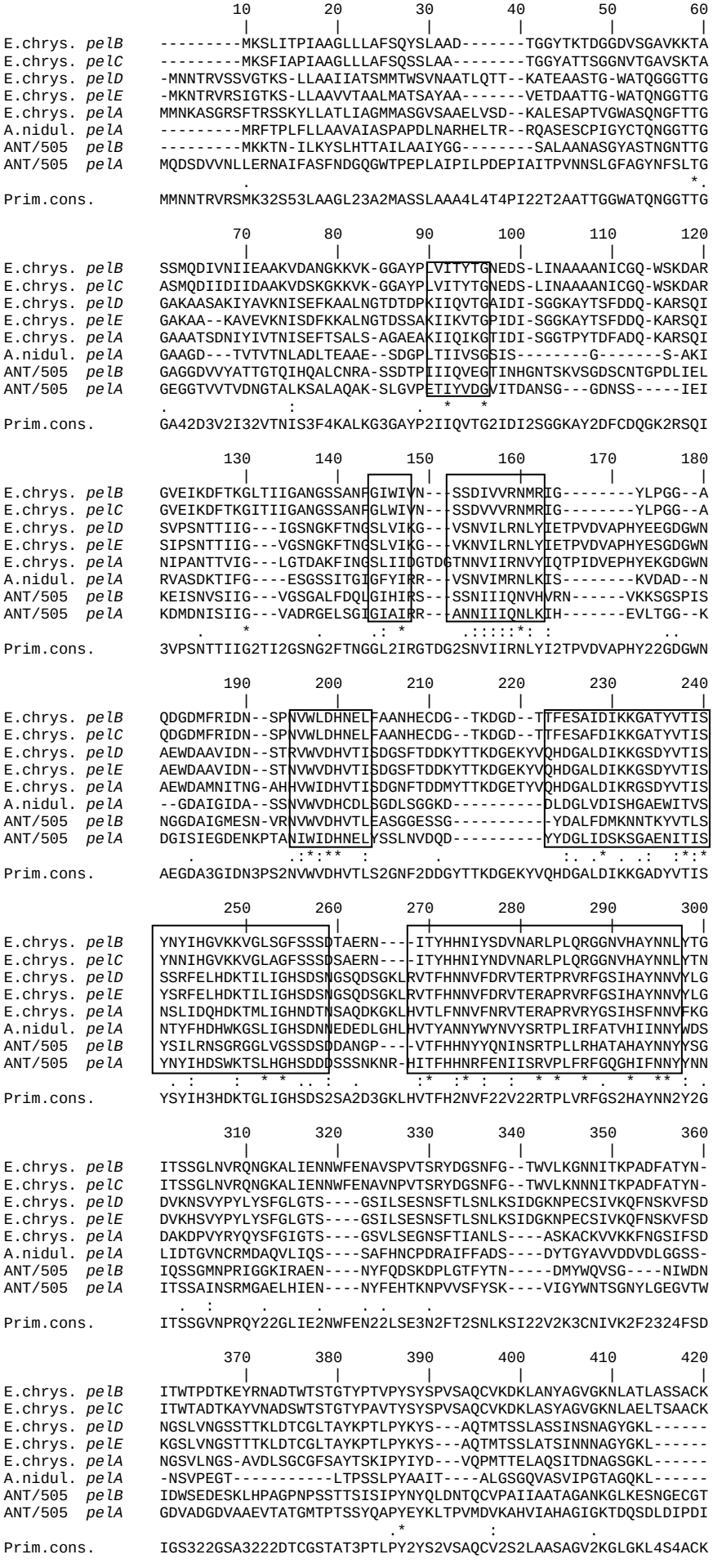
10 20 30 40 50 60
E.chrys. pelB ---------MKSLITPIAAGLLLAFSQYSLAAD-------TGGYTKTDGGDVSGAVKKTA
E.chrys. pelC ---------MKSFIAPIAAGLLLAFSQSSLAA--------TGGYATTSGGNVTGAVSKTA
E.chrys. pelD -MNNTRVSSVGTKS-LLAAIIATSMMTWSVNAATLQTT--KATEAASTG-WATQGGGTTG
E.chrys. pelE -MKNTRVRSIGTKS-LLAAVVTAALMATSAYAA-------VETDAATTG-WATQNGGTTG
E.chrys. pelA MMNKASGRSFTRSSKYLLATLIAGMMASGVSAAELVSD--KALESAPTVGWASQNGFTTG
A.nidul. pelA ---------MRFTPLFLLAAVAIASPAPDLNARHELTR--RQASESCPIGYCTQNGGTTG
ANT/505 pelB ---------MKKTN-ILKYSLHTTAILAAIYGG--------SALAANASGYASTNGNTTG
ANT/505 pelA MQDSDVVNLLERNAIFASFNDGQGWTPEPLAIPILPDEPIAITPVNNSLGFAGYNFSLTG
Prim.cons. MMNNTRVRSMK32S53LAAGL23A2MASSLAAA4L4T4PI22T2AATTGGWATQNGGTTG
70 80 90 100 110 120
E.chrys. pelB SSMQDIVNIIEAAKVDANGKKVK-GGAYPLVITYTGNEDS-LINAAAANICGQ-WSKDAR
E.chrys. pelC ASMQDIIDIIDAAKVDSKGKKVK-GGAYPLVITYTGNEDS-LINAAAANICGQ-WSKDAR
E.chrys. pelD GAKAASAKIYAVKNISEFKAALNGTDTDPKIIQVTGAIDI-SGGKAYTSFDDQ-KARSQI
E.chrys. pelE GAKAA--KAVEVKNISDFKKALNGTDSSAKIIKVTGPIDI-SGGKAYTSFDDQ-KARSQI
E.chrys. pelA GAAATSDNIYIVTNISEFTSALS-AGAEAKIIQIKGTIDI-SGGTPYTDFADQ-KARSQI
A.nidul. pelA GAAGD---TVTVTNLADLTEAAE--SDGPLTIIVSGSIS--------G-------S-AKI
ANT/505 pelB GAGGDVVYATTGTQIHQALCNRA-SSDTPIIIQVEGTINHGNTSKVSGDSCNTGPDLIEL
ANT/505 pelA GEGGTVVTVDNGTALKSALAQAK-SLGVPETIYVDGVITDANSG---GDNSS-----IEI
Prim.cons. GA42D3V2I32VTNIS3F4KALKG3GAYP2IIQVTG2IDI2SGGKAY2DFCDQGK2RSQI
130 140 150 160 170 180
E.chrys. pelB GVEIKDFTKGLTIIGANGSSANFGIWIVN--SSDIVVRNMRIG--------YLPGG--A
E.chrys. pelC GVEIKDFTKGITIIGANGSSANFGLWIVN--SSDVVVRNMRIG--------YLPGG--A
E.chrys. pelD SVPSNTTIIG---IGSNGKFTNGSLVIKG--VSNVILRNLYIETPVDVAPHYEEGDGWN
E.chrys. pelE SIPSNTTIIG---VGSNGKFTNGSLVIKG--VKNVILRNLYIETPVDVAPHYESGDGWN
E.chrys. pelA NIPANTTVIG---LGTDAKFINGSLIIDGTDGTNNVIIRNVYIQTPIDVEPHYEKGDGWN
A.nidul. pelA RVASDKTIFG----ESGSSITGIGFYIRR--VSNVIMRNLKIS--------KVDAD--N
ANT/505 pelB KEISNVSIIG---VGSGALFDQLGIHIRS--SSNIIIQNVHVRN------VKKSGSPIS
ANT/505 pelA KDMDNISIIG---VADRGELSGIGIAIRR--ANNIIIQNLKIH-------EVLTGG--K
Prim.cons. 3VPSNTTIIG2TI2GSNG2FTNGGL2IRGTDG2SNVIIRNLYI2TPVDVAPHY22GDGWN
190 200 210 220 230 240
E.chrys. pelB QDGDMFRIDN--SPNVWLDHNELFAANHECDG--TKDGD--TTFESAIDIKKGATYVTIS
E.chrys. pelC QDGDMFRIDN--SPNVWLDHNELFAANHECDG--TKDGD--TTFESAFDIKKGATYVTIS
E.chrys. pelD AEWDAAVIDN--STRVWVDHVTISDGSFTDDKYTTKDGEKYVQHDGALDIKKGSDYVTIS
E.chrys. pelE AEWDAAVIDN--STNVWVDHVTISDGSFTDDKYTTKDGEKYVQHDGALDIKKGSDYVTIS
E.chrys. pelA AEWDAMNITNG-AHHVWIDHVTISDGNFTDDMYTTKDGETYVQHDGALDIKRGSDYVTIS
A.nidul. pelA --GDAIGIDA--SSNVWVDHCDLSGDLSGGKD----------DLDGLVDISHGAEWITVS
ANT/505 pelB NGGDAIGMESN-VRNVWVDHVTLEASGGESSG----------YDALFDMKNNTKYVTLS
ANT/505 pelA DGISIEGDENKPTANIWIDHNELYSSLNVDQD----------YYDGLIDSKSGAENITIS
Prim.cons. AEGDA3GIDN3PS2NVWVDHVTLS2GNF2DDGYTTKDGEKYVQHDGALDIKKGADYVTIS
250 260 270 280 290 300
E.chrys. pelB YNYIHGVKKVGLSGFSSSDTAERN---ITYHHNIYSDVNARLPLQRGGNVHAYNNLYTG
E.chrys. pelC YNNIHGVKKVGLAGFSSSDSAERN---ITYHHNIYNDVNARLPLQRGGNVHAYNNLYTN
E.chrys. pelD SSRFELHDKTILIGHSDSNGSQDSGKLRVTFHNNVFDRVTERTPRVRFGSIHAYNNVYLG
E.chrys. pelE YSRFELHDKTILIGHSDSNGSQDSGKLRVTFHNNVFDRVTERAPRVRFGSIHAYNNVYLG
E.chrys. pelA NSLIDQHDKTMLIGHNDTNSAQDKGKLHVTLFNNVFNRVTERAPRVRYGSIHSFNNVFKG
A.nidul. pelA NTYFHDHWKGSLIGHSDNNEDEDLGHLHVTYANNYWYNVYSRTPLIRFATVHIINNYWDS
ANT/505 pelB YSILRNSGRGGLVGSSDSDDANGP---VTFHHNYYQNINSRTPLLRHATAHAYNNYYSG
ANT/505 pelA YNYIHDSWKTSLHGHSDDDSSSNKNR-HITFHHNRFENIISRVPLFRFGQGHIFNNYYNN
Prim.cons. YSYIH3HDKTGLIGHSDS2SA2D3GKLHVTFH2NVF22V22RTPLVRFGS2HAYNN2Y2G
310 320 330 340 350 360
E.chrys. pelB ITSSGLNVRQNGKALIENNWFENAVSPVTSRYDGSNFG--TWVLKGNNITKPADFATYN-
E.chrys. pelC ITSSGLNVRQNGKALIENNWFENAVNPVTSRYDGSNFG--TWVLKNNNITKPADFATYN-
E.chrys. pelD DVKNSVYPYLYSFGLGTS----GSILSESNSFTLSNLKSIDGKNPECSIVKQFNSKVFSD
E.chrys. pelE DVKHSVYPYLYSFGLGTS----GSILSESNSFTLSNLKSIDGKNPECSIVKQFNSKVFSD
E.chrys. pelA DAKDPVYRYQYSFGIGTS----GSVLSEGNSFTIANLS----ASKACKVVKKFNGSIFSD
A.nidul. pelA LIDTGVNCRMDAQVLIQS----SAFHNCPDRAIFFADS----DYTGYAVVDDVDLGGSS-
ANT/505 pelB IQSSGMNPRIGGKIRAEN----NYFQDSKDPLGTFYTN-----DMYWQVSG----NIWDN
ANT/505 pelA ITSSAINSRMGAELHIEN----NYFEHTKNPVVSFYSK----VIGYWNTSGNYLGEGVTW
Prim.cons. ITSSGVNPRQY22GLIE2NWFEN22LSE3N2FT2SNLKSI22V2K3CNIVK2F2324FSD
370 380 390 400 410 420
E.chrys. pelB ITWTPDTKEYRNADTWTSTGTYPTVPYSYSPVSAQCVKDKLANYAGVGKNLATLASSACK
E.chrys. pelC ITWTADTKAYVNADSWTSTGTYPAVTYSYSPVSAQCVKDKLASYAGVGKNLAELTSAACK
E.chrys. pelD NGSLVNGSSTTKLDTCGLTAYKPTLPYKYS---AQTMTSSLASSINSNAGYGKL------
E.chrys. pelE KGSLVNGSTTTKLDTCGLTAYKPTLPYKYS---AQTMTSSLATSINNNAGYGKL------
E.chrys. pelA NGSVLNGS-AVDLSGCGFSAYTSKIPYIYD---VQPMTTELAQSITDNAGSGKL------
A.nidul. pelA -NSVPEGT----------LTPSSLPYAAIT----ALGSGQVASVIPGTAGQKL------
ANT/505 pelB IDWSEDESKLHPAGPNPSSTTSISIPYNYQLDNTQCVPAIIAATAGANKGLKESNGECGT
ANT/505 pelA GDVADGDVAAEVTATGMTPTSSYQAPYEYKLTPVMDVKAHVIAHAGIGKTDQSDLDIPDI
Prim.cons. IGS322GSA3222DTCGSTAT3PTLPY2YS2VSAQCV2S2LAASAGV2KGLGKL4S4ACK

**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.

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

The screening of approximately 15000 clones of a genomic library of strain ANT/505 in *E. coli* DH5α 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 of 1671 and 1968 nt corresponding to proteins of 68 and 75 kDa, which were named as 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, Gold et al. 1988); and *Aspergillus nidulans* (Ho, Whitehead 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 and Hugouvieux-Cotte-Pattat 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 unusual 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 the overproduction of both pectate lyases in *E. coli* (DE3) BL21, the expression system was induced by the addition of 1 mM IPTG at 30 °C followed by a further cultivation of approximately 5 h at 30°C. After the 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 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 6 amino acids. For PelA a 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 theoretically molecular weight of 75 kDa from PelB deduced from its amino acid sequence. The N-terminal sequencing of these fragments showed in all cases AIYGGS, which is consistent with the theoretically deduced amino acid sequence of PelB. These N-terminal amino acid sequences revealed that the signal peptides of both pectate lyases were correctly processed in *E. coli*.

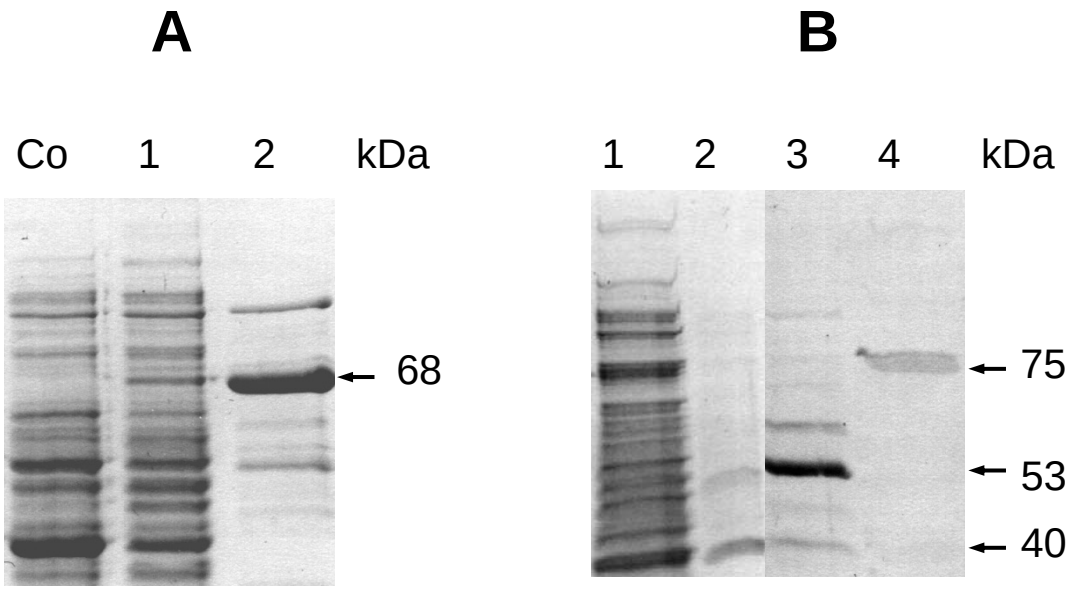


**Fig. 4** SDS-PAGE of the pectate lyases of strain ANT/505 after overproduction with the T7 expression system in *E. coli* BL21 and after purification.
(**A**) PelA; Co: *E. coli* (DE3) BL21 control without overexpression; 1: periplasmatic protein fraction of *E. coli* BL21 cells after overproduction 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

For the enzyme characterization, the purified protein fractions were used. The maximal enzymatic activities of both pectate lyases were found at a temperature of 30 °C (Fig. 5A). The remaining pectate lyase activity at 5 °C was for PelA 25 % and for PelB 35 %. The optimal enzyme activity of PelA and PelB could be found at a pH range of 9-10 (Fig. 5B). This is a typical pH of pectate lyases (Whitaker 1990). Both enzymes revealed a higher enzymatic activity with pectate than with pectin (data not shown). The Km values of both lyases for pectate and citrus pectin were 1 g/l and 5 g/l, respectively. PelA showed a higher stability at 30 °C than

PelB (Fig. 6). While PelA did not show a decrease in the enzymic activity at 30 °C over 12 h (Fig. 6A), the half life of PelB at 30°C was approximately 4 h (Fig. 6B).

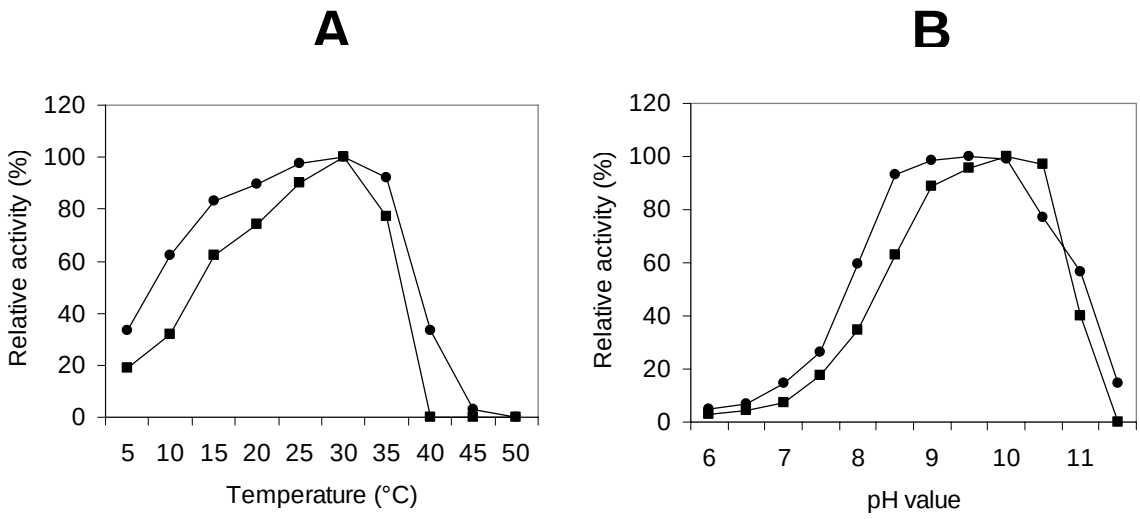


**Fig. 5** Effect of temperature (**A**) and pH (**B**) on the activity of the purified pectate lyase PelA (-●-) and PelB (-■-).

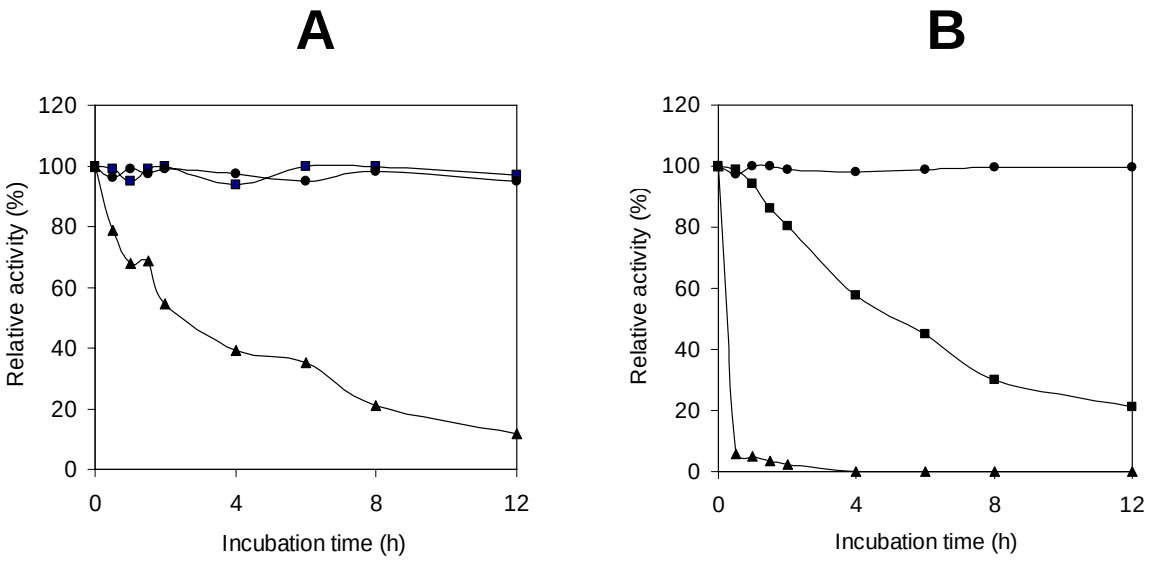


**Fig. 6.** Thermostability of the pectate lyases (**A**) PelA and (**B**) PelB at 40 °C (-▲-), 30 °C (-■-) and 20 °C (-●-).

A typical feature of pectate lyases is the dependence of their enzymatic activity on the presence of $Ca^{2+}$ (Whitaker 1990). PelA and PelB required 0.14-1 M $Ca^{2+}$ for optimal activity on pectic substrates (Fig. 7A), while 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.

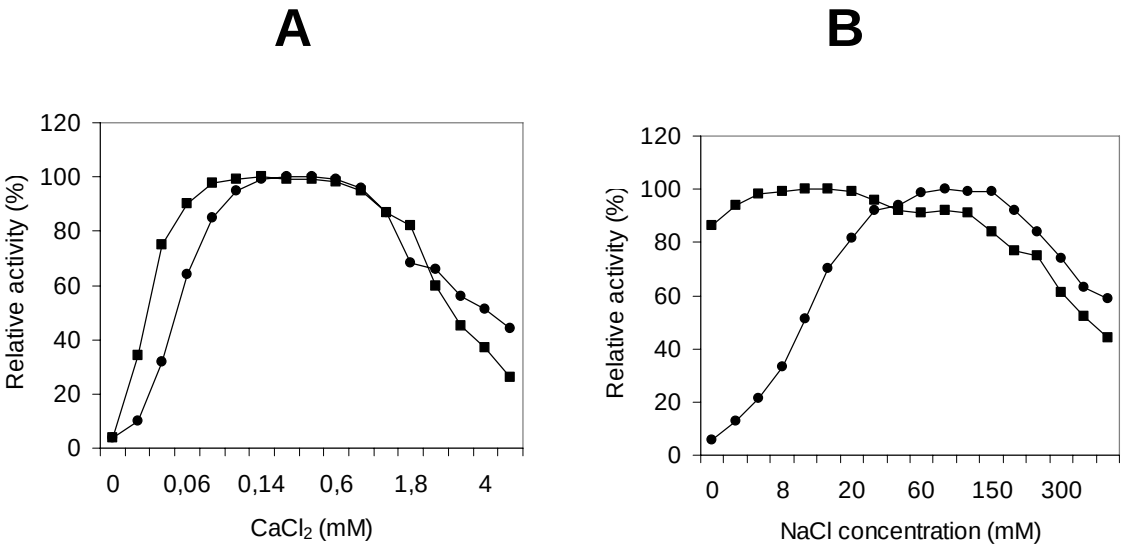


**Fig. 7** Dependence of the enzymatic activities of PelA (-●-) and PelB (-■-) on the presence of (**A**) $Ca^{2+}$ and (**B**) $Na^+$.

## Discussion

Little is known about pectin degrading marine microorganisms. Up 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 permanent cold environment like the Antarctic Ocean. According to the 16S rRNA 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 like succinate, citrate or glycerol utilization. Similar differences in catabolic activities could be found between two subspecies of *P. haloplanktis* (Gauthier, Gauthier et al. 1995).

The sequencing of the five pectinolytic clones of a 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, the dependence of the enzymatic activities on an alkaline pH and the presence of $Ca^{2+}$ (Whitaker 1990). The activity of the pectate lyase PelA of strain ANT/505 was dependent on sodium, other monovalent cations like $K^+$ could not replace $Na^+$. A similar dependence on sodium have 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 ß-elimination (data not shown), which is the typical reaction of lyases (Collmer and et 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 (Bauer and Collmer 1997; Shevchik, Robert-Baudouy et al. 1997; Tardy, Nasser et al. 1997; Soriano, Blanco et al. 2000; Pissavin, Robert-Baudouy et al. 1996). Recently a thermostable pectate lyase from a newly isolated thermophilic bacterium, from *Thermoanaerobacter italicus*, has been isolated and characterized (Kozianowski, Canganella et al. 1997). Laurent et al. (Laurent, Buchon 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 form 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, Whitehead 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 due to a toxic effect of the *pel* gene product when overproduced and was closely related to a decrease of the growth rate, and to 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 an optimal aeration of the culture was ensured. A higher cell density during the 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 in *E. coli* dependent on DsbA (Humphreys, Weir et al. 1995). This periplasmic protein of *E. coli* is involved in the formation of disulfide-bonds of secreted enzymes. Also in the case of the major *E. chrysanthemi* pectate lyases it has been shown that the cysteine residues are involved in disulfide bond formation (Yoder, Keen et al. 1993). In the case of PelB from strain ANT/505 two cysteins 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, Aittaleb 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* can not be functionally expressed in *E. coli* at 37 °C. A significant amylase activity could be only found at temperatures below 25 °C. During an 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 significant lower activity for both pectate lyases could be observed. Since PelB showed a half live 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 optimal in a temperature range from 30-37 °C, a compromise between the thermostability of such cold-adapted enzymes and the optimal temperature for the expression system has to be found.

The native polyacrylamid gel of the extracellular protein fraction indicated that the isolate ANT/505 has eventually 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 due to 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 unusual long C-terminal part, which do 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, Hoschutzky et al. 1989) and to the flagellin of *Salmonella typhimurium* (Homma, DeRosier 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* (Tsujibo, Ohtsuki et al. 1997) and to *Streptomyces coelicolor* (Redenbach, Kieser et al. 1996). Since the smaller processed PelB fragments missing the C-terminus showed a similar pectinolytic activity as 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*, a processing at the N-terminus by extracellular host proteases was described (Shevchik et al. 1998). It is interesting to note that the proteolytic processing of PelI did also 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 are also not essential for enzymatic capacity.

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

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# Chapter III

# A bifunctional pectinolytic enzyme from the psychrophilic marine bacterium *Pseudoaltermonas haloplanktis* ANT/505

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Keywords: Pectate lyase, pectin methylesterase, psychrophilic, marine, *Pseudoalteromonas haloplanktis*

## Abstract

It was recently shown that the marine psychrophilic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505 produces at least two different pectinases, PelA and PelB. In this study it is revealed that PelA from *P. haloplanktis* is an exceptionally big bifunctional enzyme featuring pectate lyase and pectin methylesterase activity. The deduced amino acid sequence of the pectin methylesterase domain showed homology to group I pectin methylesterases from *Erwinia chrysanthemi* and *Erwinia carotovora*. The pectin methylesterase domain of PelA was found to show highest homology to a potential pectin methylesterase from *Saccharophagus degradans* strain MD2-40. Maximum pectin methylesterase activity of PelA was detected at a pH of 7.5 and a maximum temperature of 30°C. This cold-adapted enzyme revealed high remaining pectin methylesterase activity at low temperatures around 5°C and was quickly unstabilized at temperatures above 45°C.

## Introduction

Although pectin is of secondary importance for the marine environment, we could recently demonstrate that the marine bacterium *Pseudoalteromonas haloplanktis* ANT/505 is able to degrade this important structural constituent of plant cell walls (Truong, Tuyen et al. 2001). Pectin is essentially composed of long chains of (1,4)-α-D-polygalacturonate, which are partially methylesterified. Microbial pectin degradation is accomplished by methylesterases, which remove the methyl groups from pectin, and the depolymerases, which degrade both pectin and pectate (Whitaker 1990). It was found that bacteria produce mainly pectate lyases, which require an alkaline pH and $Ca^{2+}$ for their optimal enzyme activity. The *P. haloplanktis* strain ANT/505 also showed pectate lyase activities, as reported by Truong et al. (2001). This psychrophilic bacterium was isolated from the permanently cold environment of the Antarctic Ocean (Weyland, Rüger et al. 1970). *P. haloplanktis* ANT/505 is able to grow in a temperature range from 0 – 29 °C (Truong et al. 2001). Up to now this strain is the only pectinolytic marine bacterium reported in the literature. It is interesting to note that the recently sequenced *P. haloplanktis* strain TAC125 does not show any pectinolytic enzyme activities (Medigue, Krin et al. 2005).

It could be shown that the *P. haloplanktis* isolate ANT/505 produces at least two pectate lyases, PelA and PelB (Truong et al. 2001). The coding sequence of both enzymes could be cloned and sequenced. According to the classification of known pectate lyases (Shevchik, Robert-Baudouy et al. 1997), PelA and PelB of *P. haloplanktis* ANT/505 could be affiliated to class I of this type of pectinases.

In this study a further sequence analysis of the pectate lyase PelA gene was undertaken. It is revealed that *pelA* of *P. haloplanktis* ANT/505 encodes an exceptionally big bifunctional pectinolytic enzyme, which - beside the pectate lyase domain – also possesses a pectin methylesterase activity.

## Materials and Methods

### Bacterial strains and cultivation conditions

The marine strain *P. haloplanktis* ANT/505 used in this study was isolated from sea ice-covered surface water in the Southern Ocean of the Antarctic (Weyland, Rüger et al. 1970; Truong, Tuyen et al. 2001). This strain was cultivated at 16°C on a modified Zobell medium

as described previously (Truong, Tuyen et al. 2001). For the purification of PelA from *P. haloplanktis* strain ANT/505 the cells were precultured in 5 ml Zobell medium for 24 h. Subsequently, 1.5 ml of the culture was transferred to 150 ml Zobell medium in a 500 ml shake flask containing 0.25% citrus pectin and the cells were cultivated for two days with 200 rpm.

*Escherichia coli* DH5α was used for all cloning experiments and for the preparation of a gene library of *P. haloplanktis* ANT/505 genomic DNA. *E. coli* BL21 (DE3) (pLysS) was used for the overexpression of the domains of the bifunctional PelA enzyme. *E. coli* cells were cultivated under vigorous agitation at 37°C in Luria Bertani (LB) medium.

**Nucleic acid manipulation**

Chromosomal DNA from strain ANT/505 was prepared by phenol-chloroform extraction according to Sambrook et al. (1989). Plasmid DNA from *E. coli* was isolated and purified by the alkaline lysis procedure (Sambrook, Fritsch et al. 1989) or using the Hight Pure Plasmid Isolation Kit (Roche Diagnostics). *E. coli* cells were tranformed with plamids by electroporation according to Sambrook et al. (1989).

**Identification of the upstream sequence of the pelA gene by dot-blot hybridization**

Chromosomal DNA of strain ANT/505 was partially digested with *Sau*3A, and fragments of about 3-4 kb were isolated from a 0.8% (w/v) agarose gel with the Gel Extraction Kit (Qiagen) and inserted into a single *Bam*HI site of pUC18. The ligated DNA was electroporated into *E. coli* DH5α cells. Positive transformants were selected on LB agar plates containing ampicilin 100 µg/ml and X-gal 100 µg/ml for blue/white selection.

For the identification of clones with DNA sequences upstream of the pectate lyase domain of the *pelA* gene dot-blot hybridization was used. For this purpose a mixture of plasmids (with a concentration of 50 ng/µl) from the genomic DNA library was denatured at 95°C for 10 min. After cooling on ice, 2 µl of denaturated plasmid DNA samples were dotted on positively charged nylon membranes (Roche Diagnostics) and fixed by UV cross-linking for 1 min. The membrane was pre-hybridized in a hybridization solution containing 5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking reagent (Roche Diagnostics) for 1.5 h at 68°C. The hybridization was carried out at 68°C overnight in the same hybridization solution supplemented with a DNA probe, which covers a sequence upstream of the pectate lyase domain of *pelA*. The DNA probe for dot-blot hybridization experiments was prepared by PCR

amplification of a 600 bp long DNA fragment with the forward primer pmeF (5'-CCTTGATTACAAAGAGCC-3') and the reverse primer pmeR (5'-TGCGCCCAGTACCACATC-3') and labeled with digoxigenin (DIG)-dUTP using the PCR DIG Probe Synthesis Kit (Roche Diagnostis). After hybridization the membrane was washed twice in 2x SSC containing 0.1% SDS at room temperature (RT) for 5 min and twice in 0.2x SSC, 0.1% SDS at 68°C for 15 min. Afterwards the hybridized samples on the membrane were incubated with DIG-specific antibodies and subsequently with CDP star (Roche Diagnostics). The membrane was exposed to X-ray film (Fuji Photo Film Co.) and positive plasmid mixtures were detected. The identification of *pelA* specific plasmid sequences from plasmid mixtures that were detected positive was performed by the same procedure as described above but with single plasmid clones.

The sequence of the DNA inserts of positive plasmid clones was determined by automated fluorescence sequencing with an ABI PRISM dye terminator cycle sequencing reaction mix (Perkin Elmer) in a 377 Perkin Elmer DNA sequencer. The complete nucleotide sequence of *pelA* of *P. haloplanktis* ANT/506 reported in this paper was submitted to Genbank with the accession number AF278706.

**Cloning and expression of the complete pelA gene and domains thereof**

For cloning of the *pelA* gene a 5247 bp long PCR-fragment covering the complete coding region including a potential signal peptide sequence was amplified from genomic DNA of strain ANT/505 with the forward primer pelAf (5'-CGATGAGCTCATGCTTATTTGTGATAGT-3') and the reverse primer pelAr (5'-GCTTCGAATTCCGCACAAAGAGAAAGGAAT-'3). After restriction with *Bam*HI and *Sac*I the fragment was cloned into pRSET-A (Invitrogen), resulting in pRSETpelA. Ligated plasmids were transformed into *E. coli* BL21 (DE3) pLysS.

The potential pectin methylesterase domain (*pme*) was cloned into the T7-expression vector pET20b(+) (Novagen) in frame with the *pelB* signal peptide sequence of the vector. The 1341 bp long *pme* DNA fragment was amplified with the forward primer Pmef (5'-ATTCGAGCTCCATGCCAGCGATTGCTAGCG-'3) and the reverse primer Pmer (5'-GCTTGCGGCCGCAAATGTAACCACCAACACG-'3), and was ligated into pET20b(+) after restriction with *Sac*I and *Not*I, resulting in pETpme. This plasmid was incorporated into *E. coli* BL21 (DE3) pLysS by electroporation.

*E. coli* BL21 (DE3) pLysS cells carrying pRSETpelA or pETpme were precultured over night in 5 ml LB medium containing ampicillin (100µg/ml) and chloramphenicol (30µg/ml). Two ml of the preculture was used for inoculation of 100 ml LB medium containing ampicillin and chloramphenicol in a 500 ml shake flask. The cultures were incubated at 37°C with 230 rpm until an optical density (OD) at 600 nm of 0.4-0.5 was reached. Subsequently, the induction of the T7 promoter was initiated by addition of 1 mM (final concentration) isopropyl thiogalactoside (IPTG) and the cultivation was continued for 5 h.

**Protein isolation**

For the isolation of total cellular proteins *E. coli* or ANT/505 cells were collected by centrifugation at 8000 rpm and 5000 rpm, respectively, for 10 min. The cell pellets were resuspended in 1/10 volume of 20 mM Tris-HCl buffer (pH 8.5) with complete Mini Protease Inhibitor tablets (Roche Diagnostics). The cells were disrupted by French Press at 9500 Psi and cell debris was subsequently sedimented by centrifugation at 14000 rpm for 15 min at 4°C. The supernatant containing the soluble cellular protein fraction was collected and stored at -70°C.

For the detection of potential inclusion bodies after overexpression in *E. coli* the cell pellets were resuspended after centrifugation in 1/10 volume of 20 mM Tris-HCl buffer (pH 7.5) with 10 mM EDTA and 1% Triton X-100 at 4°C. After disruption of the cells by French Press, the suspension was centrifuged and the pellets were collected and subsequently washed for two times in the same buffer. The final pellet, is supposed to contain the insoluble protein fraction. This protein fraction was tested by one-dimensional SDS-polyacrylamide gel electrophoresis.

Proteins from inclusion bodies were solubilized (1 mg/ml) in a solubilization buffer containing 50 mM CAPS (pH 11), 0.3% N-laurylsarcosin, and 1 mM dithiothreitol (DTT) for 15 min at RT (according to the Protein Refolding Kit from Novagen). After centrifugation at 14000 rpm for 10 min the supernatant was dialyzed two times for 3 h with cold dialysis buffer (20 mM Tris–HCl (pH 8.5) and 1 mM DTT) at 4°C. Afterwards, the refolded protein mixture was further dialyzed overnight in a 20 mM Tris–HCl buffer (pH 8.5) at 4°C with two changes of the buffer.

## Enzyme purification and assays

The purification of the PelA protein fraction from *P. haloplanktis* ANT/505 or *E. coli* cell extracts after overexpression was performed as described previously (Truong, Tuyen et al. 2001).

Pectate lyase activity was measured by the determination of reducing sugars (Bernfeld 1955) or unsaturated bonds (Collmer and et 1988). 50 µl of enzyme solution was mixed with 450 µl of 0.25% pectin acid in 50 mM Tris buffer (pH 9.5) containing 20 mM NaCl and 0.1 mM $CaCl_2$. The samples were incubated at 30°C for 60 min in a water bath. For the determination of reducing sugars, 0.5 ml of dinitrosalycilic acid (DNSA) solution was added. The solution was boiled for 10 min and cooled down to room temperature. After centrifugation for 5 min at 10000 rpm, the samples were measured spectrophotometrically at 530 nm. Sample blanks were used to correct for nonenzymatic release of the reducing sugar. One unit of pectate lyase activity was defined as 1 nM of reducing sugar liberated per minute. For determination of unsaturated bonds, the reaction was stopped by addition of 0.5 ml of 0.2 M Na-citrate buffer (pH 4.0), centrifuged at 10000 rpm for 5 min and the samples were measured at 232 nm.

Pectin methyl esterase activity was determined using a spectrophotometrical assay (Wojciechowski and Fall 1996; Pilling, Willmitzer et al. 2000) with minor adaptations. The assay solution contained 300 µl of 0.4 M $KH_2PO_4$ (pH 7.0), 300 µl of 0.5% of citrus pectin (93% methylation), 30 µl of 375 mg/ml Floral-P (Sigma) in distilled water, 2 µl of *Pichia pastoris* alcohol oxidase, 70 µl of enzyme solution with a final volume of 700 µl. The samples were incubated at 35°C for 1 h. After centrifugation at 13000 rpm for 2 min, the samples were measured spectrophotometrically at 405 nm.

## Protein electrophoresis

The one-dimensional denaturing protein gel electrophoresis (SDS-PAGE) was performed with 7.5 % polyacrylamide gels in a Mini-Protean II cell apparatus (Bio-Rad) as described by Sambrook at al. (1989). After electrophoresis, the proteins were stained with colloidal Coomassie Blue G-250 stain (Serva). PelA specific protein bands were identified by MALDI-TOF mass spectrometry as described by Borriss et al. (2006).

**Western-blot analysis**

For the detection of PelA Western-blot analyses with polyclonal rabbit anti-PelA antibodies (Biogenes) were used. For this purpose the protein bands were transferred to a PVDF membrane (Roche Diagnostis) after electrophoresis with a Mini-Trans-Blot Cell (Bio-Rad) for 5 h at 4°C and 100 V with blotting buffer (40 mM glycine, 50 mM Tris, 10 % methanol). The membrane was pre-hybridized for 1 h at RT with 2.5% nonfat milk powder in TBS buffer. The polyclonal PelA antibodies were pre-adsorbed with an *E. coli* lysed cell suspension and subsequently applied to the blots for overnight at RT. After two 20 min washing steps in TBS buffer with 2.5% nonfat milk powder, the blots were incubated for 4 h with conjugated goat anti-human IgG (Sigma) in TBS buffer with 2.5% nonfat milk powder. The blots were washed three times in TBS buffer, and the bound secondary antibody was determined by incubation with a solution consisting of 0.033 % nitroblue and 0.017 % tetrazolium-5-bromo-4-chloro-3-indolylphosphate as a colour substrate (Sambrook et al. 1989).

## Results

In the study of Truong et al. (2001) a pectate lyase sequence domain of PelA (accession numbers AF278706) with a length of 1671 bp was cloned and characterized from strain *P. haloplanktis* ANT/505. However, further analyses of this DNA sequence indicated that the published *pelA* gene sequence with the pectate lyase activity represents only a part of a bigger gene. Therefore, we tried in this study to identify the missing sequences of the *pelA* gene and to elucidate the function of this enzyme.

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                                                                MLICD
SNDDAKNNILVFSEQQAQEELVKLNDLAQQAGLVLIEEFDTDTETFFSSSYKSLSDEQGS
GPLYFKSGGSAKIDDVAGQLILEGGRITIGNTLPGKESTATDSSGVGIYNLSEGFTISFD
IISHNSAGGLSLYVDNNTASQSNSVHGGASKFYGKDINESNTPAGQRFSYTYIPGEDVNT
GPDLTNIDARGILNPEIKNSYFQIRTDSAAMIVIDNLTIKTVATDVPDVEPPVEPPVQGE
IPTVELPITTDFTALTADIFSVEHQKIIDTNGDEIPMFSKTGGSVTVIDTGLELNGGRFT
LGNTTPGIETAATDTTTSGALDLSRPYQVVMDIVSISDPEGDNKFQIYVDNNSSSSSKSI
HGNDSRFYNELINSLTAGQTLTVPGKLATKNSFLQLRTETGGTVVINNLRVEYVKDPSVF
SCTDAPELYFCDDFANGDLNNWQVLAKPDNTEAPMGEFDVLDIMGNNMMRYTAGGAGGEL
ILATEEAMANVPVTGNYFVEAKIRPRQNSTTANKHIFLMARYLNAGNWYAGGLNVQNSSS
STQVEVAVSSEGSIARPVQTKSPILLGEKGAIEDGVWYTTRFEMIDDQLTVYLDGEKMGS
ATDTSYTARGLIGVFTNNRSFEIDDVKVGDPSIKPIQLTLDYKEPNWDTSTSMDPLLINV
TAIKNDGITAGTYTVTSSNEAVVSVAINGNAITLTPITVGDAMVTFVSDSDPSITRTIKV
SVAEGFVMPTADYGDLTDKVMPAIASDSQFVDTQLSITFDNAPTFGSLGEIRIYKQNDNT
LVDTLKVGKNVDVIGYTGQDRLRSVYYYPLTIEGNTLTIKPHNNVLEYGHTYRVVIGDDV
VLGAQLNGTDFIGLGDNSQWTFTTKVTMPASNQLLVDDDGVADFRTVQGALNFAMASLAK
DEQTTISVKDGVYNELLFLRNKDNVTIQGQSQDNTTIQYENYETLNGGSGGSAPIGSGTP
GGGRGVFLVEGADMLVINNLTLKNTHLRNNVDSNQAETIYFNSNGRLVANNASFISEQDT
LLLKGYTWFYNSLVAGNVDFIWGYPVTALFEDSEIRTIGDSKDGDPTKDSSGGYVLQARV
PVQTDPGFIFLNNRFTHGPGPIGNNVLDNSTYIARSGGSDTYFDNVTLINNKFDTHIATS
GWAVQGVNSQPAPNPEIATATSGWREYGSMDLAGNTLDLSARVGGYIMQDSDVVNLLERN
AIFASFNDGQGWTPEPLAIPILPDEPIAITPVNNSLGFAGYNFSLTGGEGGTVVTVDNGT
ALKSALAQAKSLGVPETIYVDGVITDANSGGDNSSIEIKDMDNISIIGVADRGELSGIGI
AIRRANNIIIQNLKIHEVLTGGKDGISIEGDENKPTANIWIDHNELYSSLNVDQDYYDGL
IDSKSGAENITISYNYIHDSWKTSLHGHSDDDSSSNKNRHITFHHNRFENIISRVPLFRF
GQGHIFNNYYNNITSSAINSRMGAELHIENNYFEHTKNPVVSFYSKVIGYWNTSGNYLGE
GVTWGDVADGDVAAEVTATGMTPTSSYQAPYEYKLTPVMDVKAHVIAHAGIGKTDQSDLD
IPDIAPPSEPPVQEPLGLPFTENFAAANTDEFFSNSYRDLSGLAGSGTPLFHRVTGAIDV
SAGQLTMTGARISIANTTPAVSTTSADASTTGLLDLSNSYQVSFKVVTVAGNTAKASKIY
VDNNTSGSANSIHGGSSKFYSVQLQDLVPGQTDTVDGLVATATSFITLRTESEATITLDE
ITIQ
```

**Fig. 1.** The complete amino acid sequence of PelA from *P. haloplanktis* strain ANT/505. The pectin methylesterase domain (*pme*) is highlighted with a grey background. The amino acid sequence downstream the *pme* domain represents the pectate lyase domain (Truong et al. 2001). The function of the sequence upstream of the *pme* domain is unknown. The potential signal peptide sequence is underlined.

```
Echrys-PME          ------------------------------------------------------------
Ecarot-PME          ------------------------------------------------------------
BP358-PME/Pel       YGTGGFAQVYPSRGNYSDYVTFNDEAMINVLELMDDVVEKRYPFDSDVLDDSFNQRIEES 900
ANT/505-PME         ------------------------------------------MPAIASDSQFVDT- 758
MD2-40-PME          G--DPAIKPVQLSLDYASPLWEAAADQDPLNVTVTAIQSDGVTADTFTAVSSDTNVVTTS 344
                                                                 : ..

Echrys-PME          ------------------------------------------------------------
Ecarot-PME          ------------------------------------------------------------
BP358-PME/Pel       ITSGIDYILKAQIKFPDGTLTAWGAQHDPYTFEPQKARAYEHASVSGQESVGIIRFLMSR 960
ANT/505-PME         ------------------------------------------------------------
MD2-40-PME          IANNVVTITPVAQGSATVTFTAGSDANRVKTIDVEIARAFVMSTTDYGDIASKVTPTVGM 404


Echrys-PME          ------------------------------------------------------------
Ecarot-PME          ------------------------------------------------------------
BP358-PME/Pel       PQTDDIKSSIRAALQWLDDVKLENIRYISGDPNNVYFVEDLNSTAWYRFYEIGTNRGIFS 1020
ANT/505-PME         -------QLSITFDNAPTFGSLGEIRIYKQNDNTLVDTLKVGKNVDVIGYTGQDRLRSVY 811
MD2-40-PME          TDANPDAHLSITFDSAPTLSGVGSIRIYNAADDSEVDVIRLTDESDALGYAGQANKRELN 464
                              :  .       .   .  ..       .
```

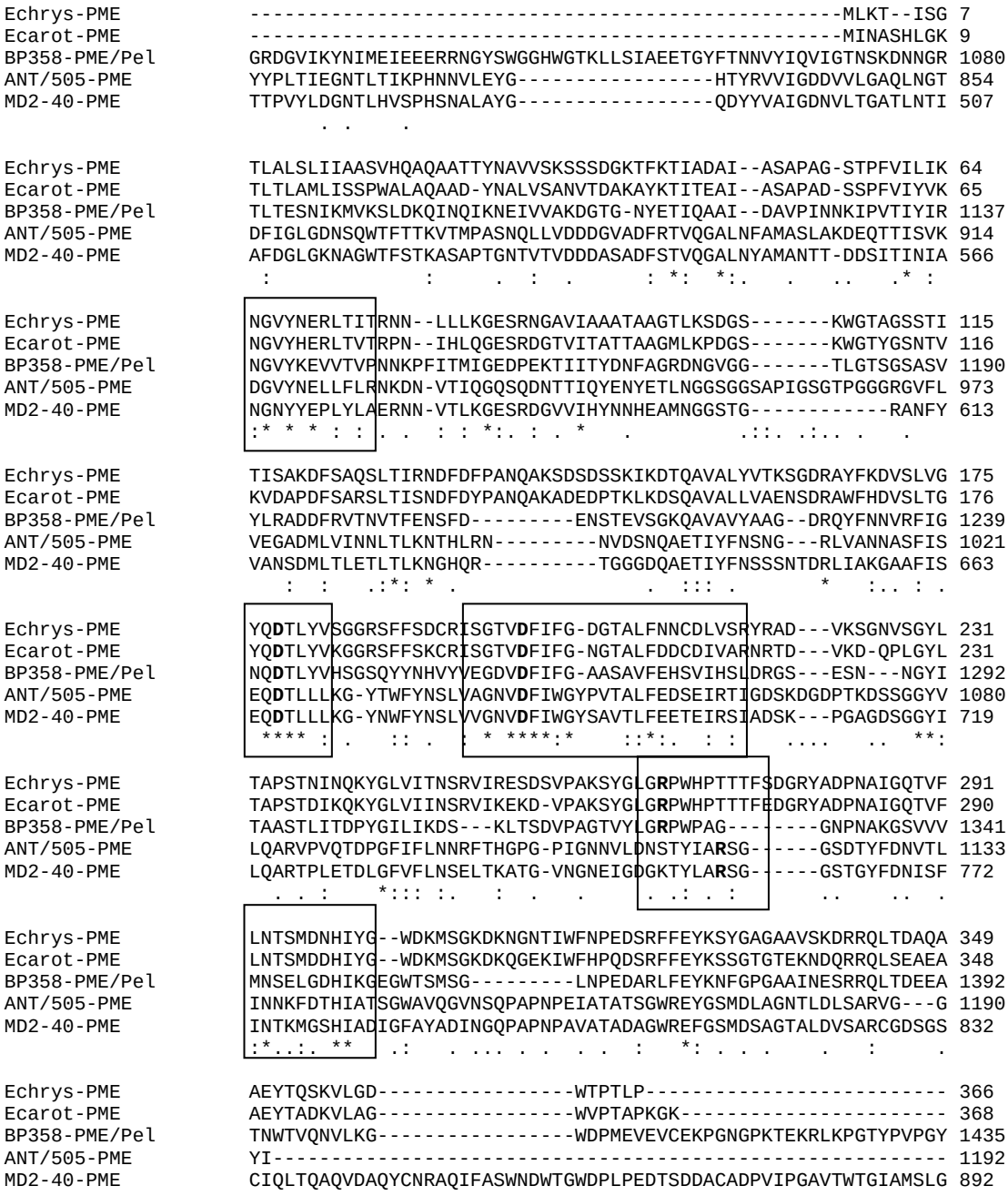


**Fig. 2.** Alignment of the amino acid sequences of the *pme* domain of *pelA* from *P. haloplanktis* ANT/505 with known pectin methylesterase sequences. Conserved regions are indicated by boxed areas. The grade of conservation of the appropriate amino acids is indicated by asterisks (highly conserved), colon (medium conserved), or a dot (low grade of conservation). The possible catalytic residues of the pectin methylesterases, which were proposed by Jenkins et al. (Jenkins, Mayans et al. 2001) are indicated by bold letters. Sequence sources aligned: Echrys-Pme from *E. chrysanthemi* (accession no. Y00549); Ecarot-Pme from *E. carotovora subsp. atroseptica* SCRI1043 (accession no. BX950801); BP358-Pme represents a bifunctional pectinolytic enzyme with separate Pel and Pme domains from an alkaliphilic *Bacillus* KSM-P358 (accession no. AB062880); MD2-40-Pme is a potential pectin methylesterase from *Saccharophagus degradans* strain 2-40 (accession no. Q21F27); ANT/505-PME represents the pectin methylesterase domain (*pme*) from PelA from *P. haloplanktis* ANT/505.

By means of dot-blot hybridisation we were able to clone the missing fragments of the *pelA* gene. Sequencing of additional DNA fragments, which were located upstream of the previously published pectate lyase sequence of PelA from *P. haloplanktis* ANT/505 (Truong et al. 2001) revealed an exceptionally long open reading frame with 5247 bp, encoding 1749 amino acids (Fig. 1). This remarkably long peptide sequence makes up a pectinolytic protein with a size of 200 kDa. Blast search analyses indicated a three domain structure of this enzyme (Fig. 1). Immediately upstream of the pectate lyase domain a potential pectin methylesterase domain could be identified by the comparison of this PelA amino acid region with known peptide sequences in the databases (Fig. 2).

The overexpression of the complete *pelA* gene in *E. coli* (Fig. 3) and the detection of the native PelA from the *P. haloplanktis* strain ANT/505 by Western-blot analysis with a polyclonal PelA specific antibody (Fig. 4) also indicated a molecular weight of PelA of about 200 kDa. The overproduction of the pme domain of PelA in *E. coli* in a soluble form could not be observed. This peptide was exclusively accumulated in form of inclusion bodies in the cytoplasm (Fig.5). The peptide upstream of the *pme* domain could not be successfully overexpressed alone or in combination with the *pme* domain in *E. coli* (data not shown). Recombinant *E. coli* cells with this DNA fragments lysed very fast, indicating that these peptides are toxic to the cells.

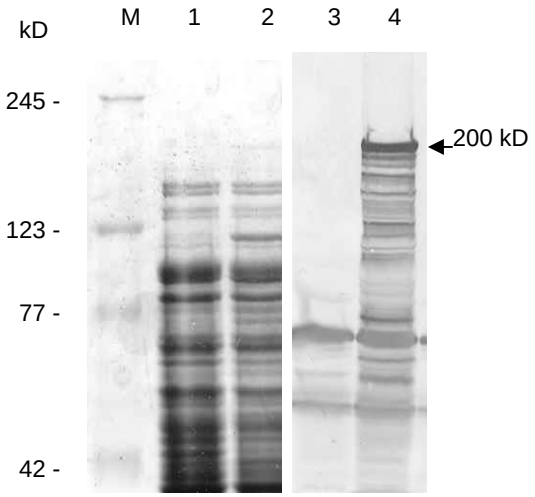


**Fig. 3.** SDS-PAGE of the cytoplasmic protein fraction of *E. coli* BL21(DE3) pLysS after overproduction of PelA with the vector pRSETpelA. (lane 1) control, pRSET without *pelA;* (lane 2) with pRSETpelA; The lanes 3 and 4 show a Western-blot of the SDS-PAGEs of lane 1 and 2 with a PelA specific polyclonal antibody. The PelA protein band at about 200 kD is indicated by an arrow.

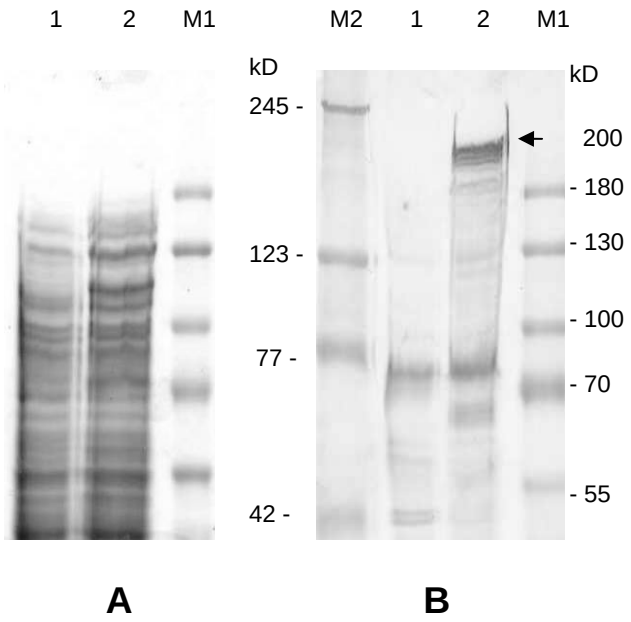


**Fig. 4.** Detection of native PelA from strain ANT/505. Cytoplasmic protein on SDS-PAGE 7.5% acrylamid (A) and Western blotting (B). Strain ANT/505 was expression in Zobell medium without pectin (lane 1) and with 0.25% of citrus pectin (lane 2). Protein marker (M1, M2). The native PelA protein band (200 kD) was indicated with *arrows*

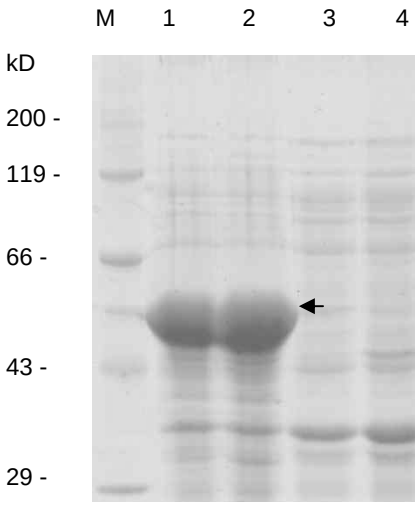


**Fig. 5.** SDS-PAGE of the inclusion body (IB) protein fraction of *E. coli* BL21(DE3) pLysS cells after overexpression of the recombinant pme domain of PelA. Lane 1 and 2: with pETpme; Lane 3 and 4: control with only pET20b(+). Odd numbers shows the IB protein fraction after a cultivation at 20°C and even numbers at 30°C. The potential pme protein domain with a calculated molecular weight of 50 kD is indicated with an *arrow*.

The potential methylesterase activity of the recombinant PelA protein and of the native PelA from *P. haloplanktis* cell extracts was tested by using an enzymatic assay on citrus pectin with a 93% methylation according to Wojciechowski and Fall (1996; Pilling et al. (2000). These enzymatic tests clearly revealed strong pectin methylesterase activity for PelA. However, the overexpressed *pme* domain revealed only a low methylesterase activity after refolding from the inclusion body fraction (data not shown). The maximal enzymatic activity was found at a temperature of 30°C (Fig. 6A). The remaining methylesterase activity of this cold-adapted enzyme at 5°C was 40%. The optimal enzyme activity could be found at a pH of 7.5 (Fig. 6B). This is a typical pH of bacterial pectin methylesterases (Whitaker 1990). The methylesterase activity of PelA showed a high thermostability at 35°C but was quickly inactivated at a temperature of 50°C, where a half life of approximately 15 min could be detected (Fig. 7).

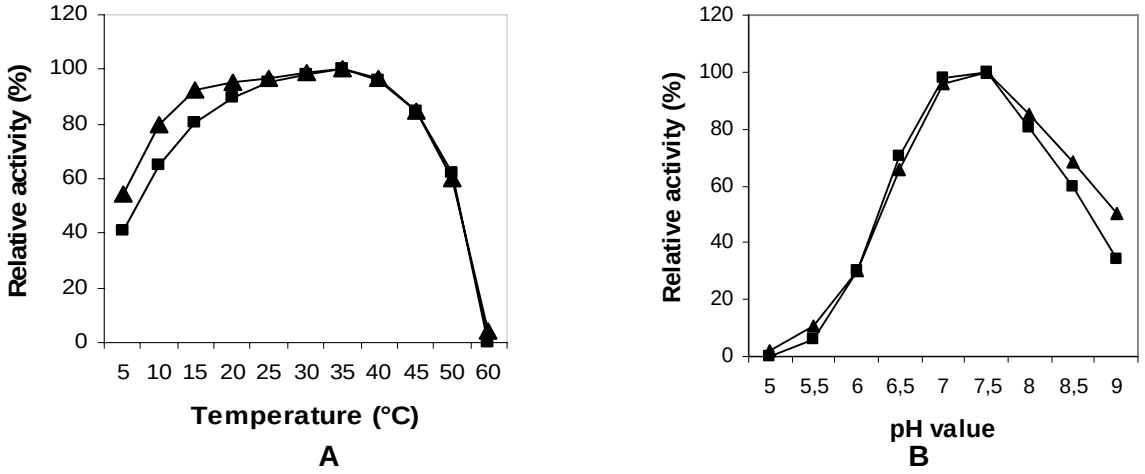


**Fig. 6.** Effect of temperature (A) and pH (B) on the pectin methylesterase activity of the purified recombinant Pme domain (*triangles*) in comparison to the native PelA protein isolated from *P. haloplanktis* ANT/505 (*squares*).

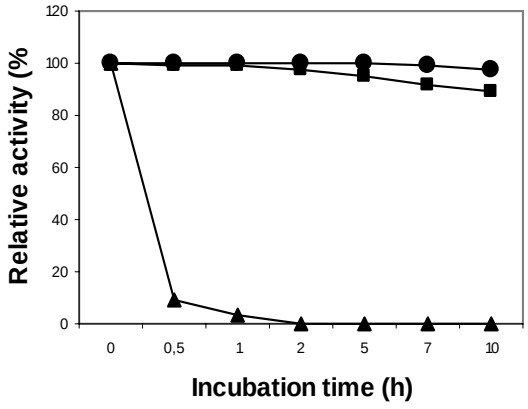


**Fig. 7**. Thermostability of the recombinant pectin methylesterase activity of PelA at 50 °C (-▲), 40 °C (-■-) and 35 °C (-●-).

A typical feature of pectate lyases is the dependence of their enzymatic activity on the presence of $Ca^{2+}$ (Whitaker 1990). It was reported that the pectate lyase domain of PelA of *P.*

*haloplanktis* requires $Ca^{2+}$ but also $Na^{+}$ for optimal activity on pectic substrates (Truong et al. 2001). However, the pectin methylesterase domain did not show a dependence on any of these ions (data not shown). In contrast, the pectate lyase activity of the recombinant PelA protein comprising both enzymatic domains showed the same dependence on sodium and calcium as the small pectate lyase domain (Fig. 8A,B). Finally, the dependency of the long native PelA enzymatic activity on temperature and pH was also comparable to the one of the pectate lyase domain alone (Fig. 9A,B).

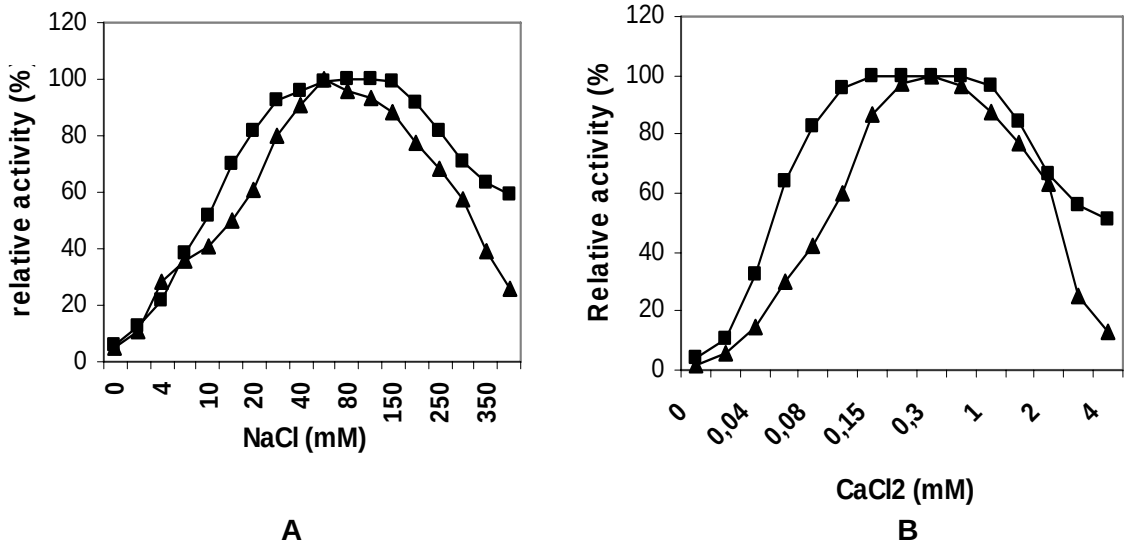


**Fig. 8.** Dependence of pectate lyase activity of recombinant PelA (*triangles*) and pectate lyase domain PelA (*squares*) on presence of Na+ (A) and Ca²+ (B).

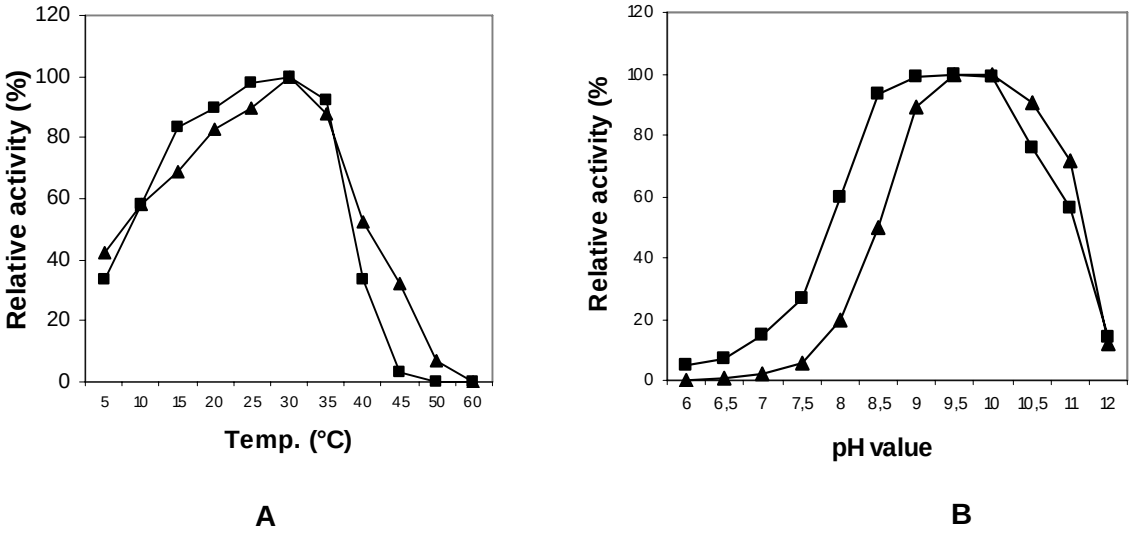


**Fig. 9**. Effect of temperature (A) and pH (B) on pectate lyase activity of purified recombinant PelA (*triangles*) and pectate lyase domain PelA (*squares*).

## Discussion

The exceptionally big pectinolytic enzyme PelA is, with a size of 200 kDa, to our knowledge the biggest pectinase reported so far. The sequence analysis of the complete *pelA* gene of the psychrophilic marine bacterium *P. haloplanktis* ANT/505 indicated a bifunctional nature of the corresponding enzyme. Blast search analyses of the PelA amino acid sequence revealed immediately upstream of the pectate lyase domain a potential pectin methylesterase domain. Several conserved regions homologous to sequences of known pectin methylestrases could be detected (Fig. 2). A sequence comparison to other protein sequences in the databases indicated an affiliation of the *pme* domain of PelA to the group 1 of pectin methylesterases. The middle part (amino acid position 746 to 1192) of the deduced amino acid sequence of PelA comprising about 447 amino acids showed 29% and 25% homology to pectin methylesterases from *E. carotovora* (accession no. BX950801) and from *E. chrisanthemi* (accession no. Y00549), both of which had been suggested to belong to the group 1 of pectin methylesterases. With 51.7% the highest homology of the *pme* domain could be found to a potential pectin methylesterase from *Saccharophagus degradans* strain MD2-40 (accession number Q21F27). Further pectin methylesterases, which revealed homologous sequences to the *pme* domain were a pectin methylesterase from *B. licheniformis* (30.24%) (accession No. Q65F39), an unclassified bifunctional pectinolytic enzyme with separate pectate lyase and *pme* domains from an alkaliphilic *Bacillus* KSM-P358 (29.4%) (accession no. AB062880), and a potential pectin methylesterase from *Clostridium* sp. (31.1%) (accession No Q4CDY1).
Pectin-degrading enzymes have to be secreted out of the cells to reach their substrate. It was found that the pectate lyase PelB of *P. haloplanktis* ANT/505 was secreted into the extracellular medium (Truong et al. 2001). However, the PelA activity could only be located in the periplasm of *P. haloplanktis*. The sequence analysis of the N-terminus of PelA revealed a potential signal peptide with a length of 22 amino acids (Fig. 1). In addition two potential membrane spanning domains could be detected in the peptide sequence regions 686:701 and 891:906 of PelA, indicating an incorporation of this enzyme into the cellular membrane.


**Fig. 10**. Alignment of N-terminal region of PelA from *P. haloplanktis* ANT/505 with Fibronectin type 3-like domains from different bacteria. Conserved regions are indicated by boxed areas. The grade of conservation of the appropriate amino acids is indicated by asterisks (highly conserved), colon (medium conserved), or a dot (low grade of conservation) Abbreviations (accession numbers are given in parentheses): Chi_Vibrfu, *Vibrio furnissii* chitinase (P96156); Chitd_Vibrcho, *Vibrio cholerae* chitodextrinase (NP_233087); Chi_Vibrcho_1, *Vibrio cholerae* chitinase VC1952 (ZP_00752148); Chi_Vibrcho_2, *Vibrio cholerae* chitinase RC385 (ZP_00752148); CbhA_Clotm_1, *C. thermocellum* cellobiohydrolase (EAM46132); CbhA_Clotm_2, *C. thermocellum* cellobiohydrolase (CAA56918); Cel_Thermfu, *T. fusca* exocellulase (YP_290015); Cel_Streptco, *S. coelicolor* cellulase (NP_630627); ANT/505_pelA, *P. haloplanktis* ANT/505 pectin methylesterase-pectate lyase PelA
Accession numbers are from Genbank database.

By means of an enzymatic assay a clear methylesterase activity of PelA and the *pme* domain could be shown. While many pectinases have been found in microorganisms, such a bifunctional enzyme seems not to be that common (Keen and Tamaki 1986; Nasser, Chalet et al. 1990). Recently, a bifunctional pectinolytic enzyme with separate pectate lyase and pectin methylesterase domains from an alkaliphilic *Bacillus* have been described Kobayashi et al. (2003). Furthermore, in a recent study Solbak et al. (2005) used a high throughput screening assay with complex environmental metagenome DNA samples and could discover more than 40 novel microbial pectate lyases. It was shown that many of these pectate lyases were modular in structure and contained not only a pectate lyase catalytic domain belonging either

to the polysaccharide lyase families 1 or 10, but had also methylesterase domains and a variety of other modules such as N- or C-terminal carbohydrate binding modules. Several bifunctional enzymes containing a pectin methylesterase domain and a pectate lyase domain from either polysaccharide lyase family 1 or 10 could be discovered in this study. Some of the pectinolytic enzymes discovered by Solbak et al. (2005) also contained fibronectin type 3-like modules. Such a domain could also be detected in the N-terminal region of PelA (Fig. 10). Although these domains are common among bacterial extracellular glycohydrolases their function has not yet been fully elucidated (Kataeva, Seidel et al. 2002).

**Acknowledgements** We gratefully acknowledge mass spectrometric analyses by Dirk Albrecht.

# Chapetr IV

# Regulation of the pectinolytic genes of the psychrophilic marine bacterium *Pseudoalteromonas haloplanktis* ANT/505

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Keywords: pectinase, regulation, psychrophilic, marine, *Pseudoalteromonas haloplanktis*

## Abstract

*Pseudoalteromonas haloplanktis* strain ANT/505 is a marine, psychrophilic bacterium, isolated from sea ice-covered surface water of the Southern Antarctic Ocean. It was recently shown that this *P. haloplanktis* strain expresses pectinolytic activities, which is very uncommon for a marine bacterium. *P. haloplanktis* ANT/505 produces at least two different pectinases: PelA, a bifunctional enzyme with pectate lyase and pectin methylesterase activity, and PelB, a pectate lyase. In this study it is shown that these pectinase genes are expressed from independent cistrons, which are not clustered but located at distant positions on chromosome I of the *P. haloplanktis* genome. It was found that the transcription of both pectinase genes is induced by the presence of pectin. By means of primer extension the promoter regions of both cistrons were detected.

## Introduction

The expression of many pectinolytic genes is tightly regulated. In most of the microbial pectin degraders, the presence of pectin is the main signal required to induce the expression of the genes involved in pectinolysis. Furthermore, the expression of pectinolytic genes is frequently a subject to other physiological controls, including growth phase–dependent induction, catabolite repression, and variations in environmental conditions; e.g. temperature, osmolarity, or nutrient starvation (Hugouvieux-Cotte-Pattat et al. 1996). The induction of pectinase expression under different environmental or growth conditions requires several independent regulatory mechanisms. Pectin lyase synthesis in *Erwinia* is controlled by a complex regulatory network involving the repressor KdgR (Reverchon, Nasser et al. 1991; Nasser, Reverchon et al. 1994) and additional regulatory proteins such as PecS and PecT (Reverchon et al. 1994, Surgey et al. 1996) but also the catabolite activator protein CRP (Nasser, Robert-Baudouy et al. 1997).

Although pectin is of secondary importance for the marine environment, it was shown that the marine bacterium *Pseudoalteromonas haloplanktis* ANT/505 is able to degrade this organic polymer of terrestrial origin (Truong et al. 2001). It was found that *P. haloplanktis* produces pectate lyases, which require an alkaline pH and $Ca^{2+}$ for their optimal enzyme activity. It could be shown that the *P. haloplanktis* isolate ANT/505 produces two pectate lyases, PelA and PelB (Truong et al. 2001). It was revealed that the PelA gene of *P. haloplanktis* ANT/505 encodes a bifunctional pectinolytic enzyme, which possesses beside the pectate lyase domain a pectin methylesterase activity (Truong et al. 2006). In this study we characterized the genomic organization of these two pectinolytic genes and their potential regulation in *P. haloplanktis*.

### Materials and Methods

#### Bacterial strains and cultivation conditions

The marine strain *P. haloplanktis* ANT/505 used in this study was isolated from sea ice-covered surface water in the Southern ocean of the Antarctic (Weyland et al. 1970; Truong, Tuyen et al. 2001). This strain was cultivated for 2 days at 16°C on a modified Zobell medium as described previously (Truong, Tuyen et al. 2001).

*Escherichia coli* DH5α was used for the preparation of a gene library of *P. haloplanktis* ANT/505 genomic DNA. *E. coli* BL21 (DE3) (pLysS) was used for the overexpression of the domains of the bifunctional PelA enzyme. *E. coli* cells were cultivated under vigorous agitation at 37°C in Luria Bertani (LB) medium.

**Nucleic acid manipulation**

Chromosomal DNA from strain ANT/505 was prepared by phenol-chloroform extraction according to Sambrook et al. (1989). Plasmid DNA from *E. coli* was isolated and purified by the alkaline lysis procedure (Sambrook, Fritsch et al. 1989) or using the Hight Pure Plasmid Isolation Kit (Roche Diagnostics). *E. coli* cells were tranformed with plamids by electroporation according to Sambrook et al. (1989).

**Identification of the upstream sequence of the pelB gene by dot-blot hybridisation**

For the identification of the upstream sequence of *pelB* suitable clones were isolated from a genomic DNA-library of *P. haloplanktis* ANT/505 by dot-blot hybridization as described in chapter III. The *pelB* specific DNA probe for dot-blot hybridization experiments was prepared by PCR amplification of a 600 bp long DNA fragment with the forward primer "pelB-upper F" and the reverse primer "pelB-upper R" (Tab. 1).
The sequence of the DNA inserts of positive plasmid clones was determined by automated fluorescence sequencing with an ABI PRISM dye terminator cycle sequencing reaction mix (Perkin Elmer) in a 377 Perkin Elmer DNA sequencer. The upstream nucleotide sequence of *pelB* of *P. haloplanktis* ANT/506 reported in this paper was submitted to Genbank with the accession number AF278706. The Fasta 3 and ClustalW multi alignment programmes were used for the alignment of the open reading frames of *P. haloplanktis* strain ANT/505 with known sequences from *P. haloplanktis* strain TAC125.

**Table 1.** List of primer used in this study. *The T7 promoter sequence is underlined.

| Name | Sequences* | Purpose |
|---|---|---|
| pelAF1 | TGATTGTCATTGATAAC | RNA probe for the unknown domain of *pelA*. |
| pelAR1-T7 | CTAATACGACTCACTATAGGGAGATA CAGATGGATCTTTAAC | |
| pelAF2 | TTGATTACAAAGAGCCT | RNA probe for the *pme* domain of *pelA* |
| pelAR2-T7 | CTAATACGACTCACTATAGGGAGATG CGCCCATCCGTGAGTT | |
| pelAF3 | TTAGCACAAGCAAAATCA | RNA probe for the *pel* domain of *pelA* |
| pelAR3-T7 | CTAATACGACTCACTATAGGGAGATT GGCTATTATCGACCGAGG | |
| pelBF | ATGAAAAAAACTAATATT | RNA probe for *pelB* |
| pelBR-T7 | CTAATACGACTCACTATAGGGAGATC GACCTGAATTACGCAA | |
| pelAR-PE | TGAGTTTGACTAACTCTTCTTG | primer extension *pelA* |
| pelBR-PE | CAGCTAGGGCACTGCCGCCATA | primer extension *pelB* |
| pelB-upper F | ATGAAAAAAACTAATATT | *pelB* upstream sequence cloning |
| pelB-upper R | TCGACCTGAATTACGCAA | |

## Northern- and slot-blot analyses

Total RNA from *P. haloplanktis* ANT/505 cells was isolated by a method as described by Jürgen et al. (2005). For Northern-blot hybridization and slot-blot experiments, specific digoxigenin-labelled RNA probes for the *pelA* and *pelB* coding sequences were produced with the primers listed in Table 1 by in vitro transcription with a T7 RNA polymerase from internal PCR products containing a T7 promoter sequence by using the Dig-labeling Kit from Roche Diagnostics as described by Schweder et al. (1999).

For the analysis of the pectinolytic gene expression at the transcriptional level the ANT/505 cell samples were taken throughout the growth phase at $OD_{600nm}$ of 1.6, 1.8 and 2.1 (Fig. 1). The Northern-blot analysis was performed as described by Nickel et al.(2004). In brief, total RNA was separated by electrophoresis in a 0.6% agarose gel containing 2.1 M formaldehyde-MOPS (morpholinepropanesulfonic acid). The gels were stained with ethidium bromide, and the separated RNA was subsequently transferred to a positively charged nylon membranes (Roche Diagnostics) with 20x SSC by using a vacuum blotter. For the slot-blot analyses total RNA was directly transferred to positively charged nylon membranes with vacuum using a Hoefer PR648 slot-blot apparatus (Amersham Pharmacia Biotech) as described by Sambrook, et al. (2001). After backing at 120°C for 1h, the membranes were hybridized with RNA-labelled probes overnight at 68°C in hybridization buffer (5x SSC, 0.1% N- lauroylsarcosin Na-salt, 7% SDS, 2% Blocking reagent and 50% formamid). The membranes were washed twice at room temperature for 5 min and at 68°C for 15 min in 0.1 x SSC with 0.1% SDS. The RNA blots were analysed in the LumiImager system from Roche Diagnostics.

**Primer extension analysis**

The primer extension was performed as described by Wetzstein et al. (1992). The primers used in these experiments are listed in the Table 1. The primers complementary to the regions immediately down stream of the potential start codons of *pelA* or *pelB* were 5' end labeled with ($\gamma$- $P^{32}$)-ATP by using T4 polynucleotide kinase. Labeled primers of *pelA* and *pelB* were annealed to10 µg and 5 µg of total RNA isolated from ANT/505 cells at late log phase respectively. The primer extension reaction was done by using reverse transcriptase (Roche Diagnostics) and products were separated by electrophoresis on a 6% polyacrylamide sequencing gel. Didesoxynucleotide sequencing reactions (Sambrook, Fritsch et al. 1989) using the same primer and an appropriate plasmid DNA template were run in parallel to allow determination of the end points of the extension products.

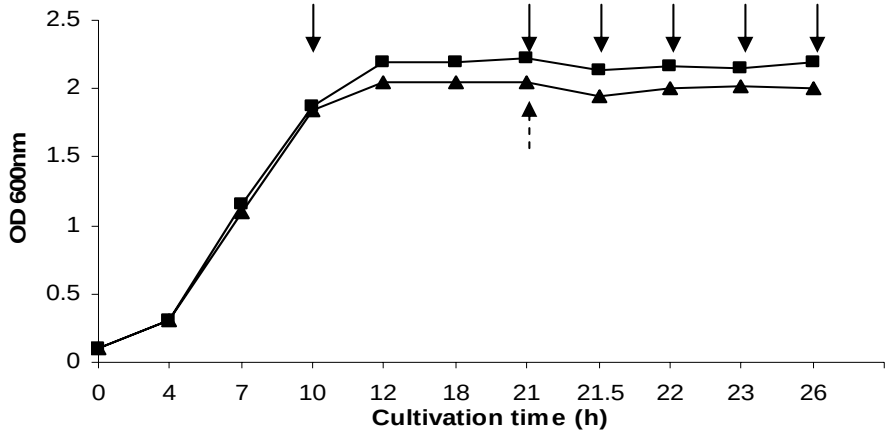


**Fig. 1.** Growth curve of *P. haloplanktis* strain ANT/505 in Zobell medium without pectin (triangles) and 0.25% pectin (squares). The sampling is indicated by arrows with solid lines, the addition of pectin to the medium is indicated by an arrow with a dotted line.

## Results

In the study of Truong et al. (2001) a pectate lyase sequence domain of PelA with a length of 1671 bp was cloned and characterized. In a recent study it was demonstrated that the *pelA* gene sequence is much bigger and codes for a bifunctional enzyme with at least two enzymatic domains, a pectate lyase and a methylesterase domain with a third N-terminal domain of unknown function (Truong et al. 2006). In order to prove that these different domains of *pelA* are indeed encoded by the same transcript, three different RNA probes specific to these domains were prepared. By Northern-blot experiments it could be verified that these three domains are all located on the 5247 bp long *pelA* gene (data not shown). In Figure 2 one exemplary Northern-blot with the RNA probe specific for the *pme* domain is shown, revealing a 5.8 kb long specific *pelA* transcript. The second RNA band at 2.37 kb visible in the Northern-blot is most probably a degradation product of the *pelA* mRNA. In the stationary phase the long *pelA* transcript turned out to be very unstable. Northern-blot analysis of the *pelB* mRNA showed a 2.1 kb long transcript (Fig.3).

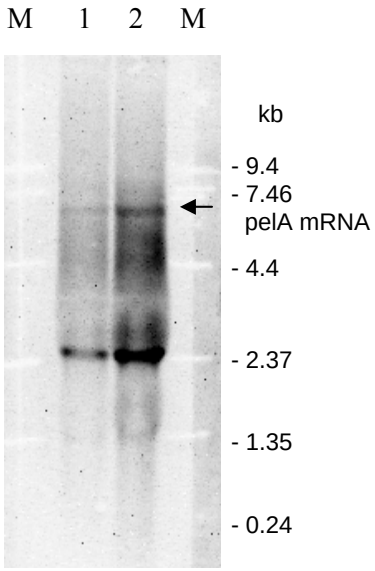


**Fig 2.** Northern-blot analysis of the *pelA* mRNA with the RNA probe homologue to the *pme* domain. Total RNA was isolated from ANT/505 cells at the late log phase corresponding to an OD (600 nm) of 1.8. Lane 1: 10 µg of total RNA, lane 2: 20 µg of total RNA, M: RNA marker. The *pelA* mRNA transcript with a size of 5.74 kb is indicated with an arrow.

The determination of the pectate lyase activity in *P. haloplanktis* ANT/505 cell cultures showed a significant increase of the pectinolytic activity throughout the growth curve in Zobell medium with citrus pectin or pectate (Truong et al. 2001). In order to elucidate whether pectin induces the expression of the pectinolytic genes in *P. haloplanktis* ANT/505 at the transcriptional level, we analyzed the mRNA levels of *pelA* and *pelB* from cells grown without (ZB1) or with citrus pectin from the beginning of the cultivation (ZB2). The growth curves of ANT/505 cells under these conditions are shown in Figure 1. No significant differences in the growth rates of these different cell cultivations could be detected. However, the cultures with pectin always reached a higher final optical density. Slot-blot analyses using *pelA* and *pelB* specific RNA probes showed a high mRNA level of both genes after 10 h of cultivation if pectin was added from the beginning of the cultivations (Fig. 4). In comparison, the cultures without pectin did not show any *pelA* or *pelB* expression at that time. After 21 h the cells in the pectin-containing ZB2 cultures revealed only a low remaining *pelA* and *pelB* mRNA level. However, already 30 min after addition of new pectin to the medium a clear induction of the *pelA* and *pelB* expression could be observed in these cultures. Interestingly, also in the ZB1 cultures, where no pectin was supplemented at the beginning of the

cultivations, a clear induction of the *pelA* and *pelB* expression could be observed (Fig. 4). Here the maximum induction could be detected only 2 h after addition of 0.25% citrus pectin. Five hours after addition of pectin (which was applied 26 h after the start of the cultivation) a down-regulation of the *pelA* and *pelB* expression in all cultivations could be detected.

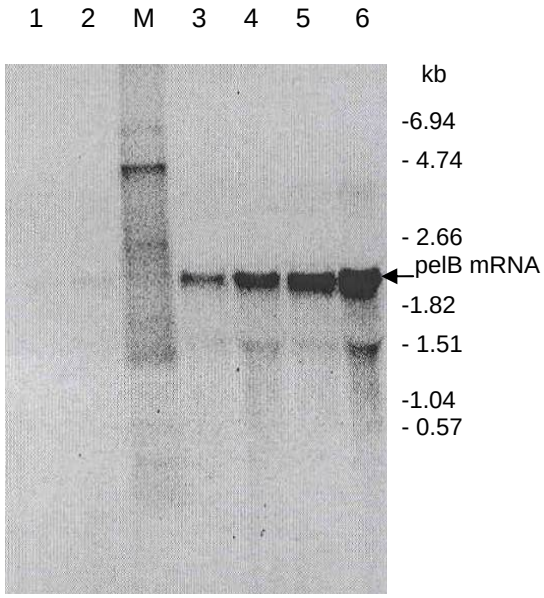


**Fig 3.** Northern-blot analysis of the *pelB* mRNA. Total RNA was isolated from ANT/505 cells at different time points throughout the growth curve corresponding to an OD (600 nm) of 1.6 (lane 1, 2); 1.8 (lane 3, 4) and 2.1 (lane 5, 6). Lane 1, 3, 5: 4 µg of total RNA; lane 2, 4, 6: 8 µg of total RNA. The *pelB* mRNA transcript with a size of 2.2 kb is indicated with an arrow. M: RNA standard

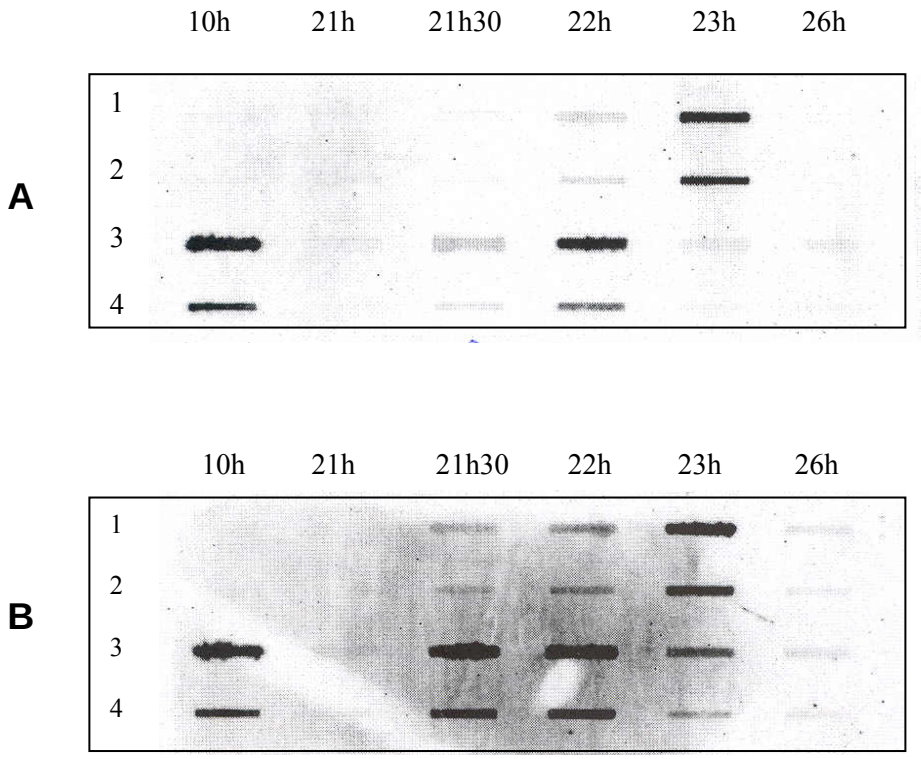


**Fig. 4.** Slot-blot analysis of the *pelA* (A) and *pelB* (B) mRNA levels of strain ANT/505 during growth in Zobell medium without pectin (ZB1) (lanes 1, 2) or with 0.25% of citrus pectin ZB2) (lanes 3, 4) added from the beginning of the cultivation. For the detection of the *pelA* transcript the RNA probe specific to the *pme* domain was used. After 21 h cultivation in both case 0.25% citrus pectin (final concentration) was added to the medium. Lane 1 and 3: 10 µg of total RNA per slot; lane 2 and 4: 5 µg of total RNA per slot.

The start points of transcription of the *pelA* and *pelB* gene in strain ANT/505 were determined by primer extension analyses (Fig. 5). By this procedure, similar expression profiles could be observed for *pelA* (Fig. 5A) and *pelB* (Fig. 5B) at the transcriptional level, using RNA samples isolated from the cells grown on Zobell medium with pectin. The transcription initiation site for *pelA* was found 30 nucleotides upstream of its translational start codon (Fig. 5A, Fig. 6). The sequences TATAGT at position -10 and CTCATAA at position -35 are suggested as putative promoter motives according to (Rosenberg and Court 1979) for *pelA* (Fig. 6). The primer extension suggested a transcriptional start site for *pelB* 34 nucleotides upstream of its translational start codon ATG (Fig. 5b, Fig. 7). A potential promoter sequence at -10 (TAAAGT) and at -35 (TTGTTAT) is suggested for *pelB* in Figure 7.

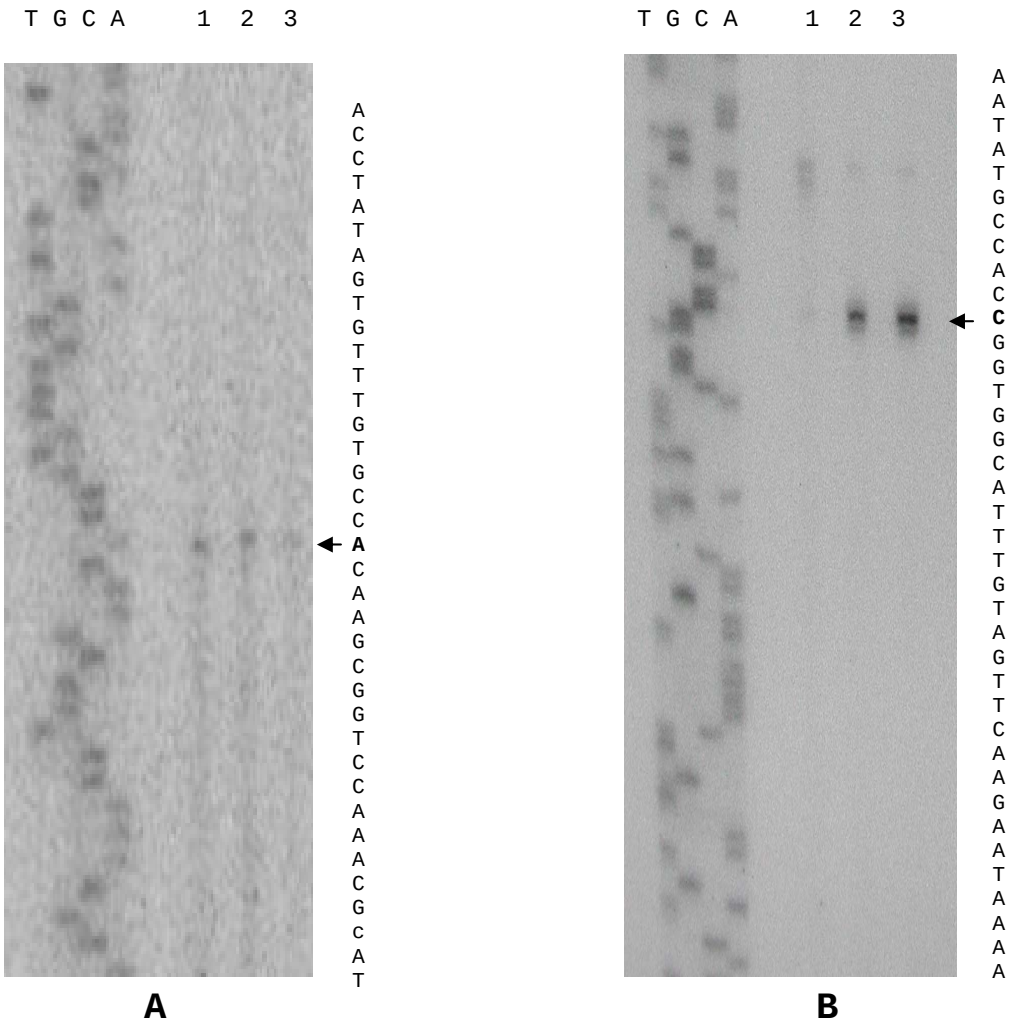


**Fig. 5.** Primer extension analysis of the start point of the *pelA* (A) and *pelB* (B) transcripts. Total RNA was isolated from *P. haloplanktis* ANT/505 cells at different time points throughout the growth phase corresponding to OD (600 nm) of 1.6 (lane 1); 1.8 (lane 2) and 2.1 (lane 3). The potential transcriptional start sites are indicated by arrows.

```
gatctcaggtgaaggtgaactgctacaacccagtatattatttctcgctctcataatacg
                                                      -35
gttaataacacctatagtgtttgtgccacaagcggtccaaacgcattggcaacactggtg
             -10         +1                    RBS       M
cttatttgtgatagtaatgatgatgctaaaaacaatattttagtgttctccgaacaacaa
 L  I  C  D  S  N  D  D  A  K  N  N  I  L  V  F  S  E  Q  Q
gcccaagaagagttagtcaaactcaacgatttagctcagcaagctggtttagtcttaatt
 A  Q  E  E  L  V  K  L  N  D  L  A  Q  Q  A  G  L  V  L  I
gaagagtttgatactgacactgaaacatttttctcatcgagctataaaagcttatcggat
 E  E  F  D  T  D  T  E  T  F  F  S  S  S  Y  K  S  L  S  D
gagcaaggcagcggcccgttatattttaaaagtggcggtagcgcaaaaattgacgatgta
 E  Q  G  S  G  P  L  Y  F  K  S  G  G  S  A  K  I  D  D  V
gcaggccaactaattcttgaaggcggccgtattaccatcggtaataccctgcccggcaaa
 A  G  Q  L  I  L  E  G  G  R  I  T  I  G  N  T  L  P  G  K
gaatcaacagcgactgatagctcaggtgtggggatttataatttaagcgaagggtttacc
 E  S  T  A  T  D  S  S  G  V  G  I  Y  N  L  S  E  G  F  T
attagctttgatattatttctcataatagtgctggtggattatccctgtatgttgataat
 I  S  F  D  I  I  S  H  N  S  A  G  G  L  S  L  Y  V  D  N
```

**Fig. 6.** Nucleotide and deduced N-terminal amino acid sequences of the *pelA* gene of *P. haloplanktis* strain ANT/505. The mRNA starting point is indicated by "+1". The potential promoter region at positions -35 and -10 and the ribosomal binding site are underlined.

```
actcggttttagttaaagtcacctgaactttggataataaatcctttaaccagggttcag
gttgaataaatccctacatctgcactattttaacatcagaaaataaaaggcaatttcact
aagtaacctgaactctggataataaatttaaatatcaccataactcaatacgttagtaaa
cttcttaaccggggttcagattaagtaacaattccatttttagttttttcagttaaacag
tactgcttaaaccaacctgcataaatttttgagcaatttaacaaattaaattgcccctgg
gcaaaaccgtacgagcagcgcatgtttaccatttattctacgttatcgcctatttatgga
gaataaccatactacataggccctgtcttgtctaaacaccaagcttactgctgtaagttc
agcctcgaaagataaacaggcccaaaatgaagtatttcttaatcgattacaaaaaaaata
ctgtgattacaaatatacacactttcttaaatgcatgatatttatatgttattttattgt
aaattaatcattacaaaaaagatcgattcatctaatccttttttacttattgttattatta
                                                      -35
ttatttaaagtgaatatgccaccggtggcatttgtagttcaagaataaaacttgttaatg
      -10           +1                  RBS              M
atcactgcagtcttaaatacaaaagtttttacactgaaaaccatttttaattggagttaaa
 I  T  A  V  L  N  T  K  V  F  T  L  K  T  I  L  I  G  V  K
atgaaaaaaactaatattttaaaatactctttacacacaactgcaatattagccgcaatt
 M  K  K  T  N  I  L  K  Y  S  L  H  T  T  A  I  L  A  A  I
tatggcggcagtgccctagctgcaaatgcatctggatatgcttcaacaaacggtaataca
 Y  G  G  S  A  L  A  A  N  A  S  G  Y  A  S  T  N  G  N  T
acaggtggtgccggtggtgatgtagtatatgcaacaacaggaacccaaatacaccaagct
 T  G  G  A  G  G  D  V  V  Y  A  T  T  G  T  Q  I  H  Q  A
ttatgtaatcgtgcatcgagtgatacaccaattatcattcaagttgaaggcacaattaat
 L  C  N  R  A  S  S  D  T  P  I  I  I  Q  V  E  G  T  I  N
catggtaatacaagcaaggtatctggtgatagctgtaacactggtcctgacttaattgaa
 H  G  N  T  S  K  V  S  G  D  S  C  N  T  G  P  D  L  I  E
```

**Fig.7.** Nucleotide and deduced N-terminal amino acid sequences of the *pelB* gene of *P. haloplanktis* strain ANT/505. The mRNA starting point is indicated by "+1". The potential promoter region at positions -35 and -10 and the ribosomal binding site are underlined.

In order to check the potential position of *pelB* on the ANT/505 chromosome, a DNA fragment upstream of the coding sequence of this gene was cloned by slot-blot hybridization from an ANT/505 genomic library. Approximately 3000 transformants were screened, which led to the identification of a 4 kb fragment corresponding to the N-terminal coding sequence of *pelB*. DNA sequence analysis showed an open reading frame (orf) of 1739 bp and a partial orf of 629 bp downstream of the coding sequence of *pelB* in *P. haloplanktis* ANT/505. Blast search analyses with the *P. haloplanktis* TAC125 genomic DNA identified these two sequences as *cydC* and *cydB* coding for two cystein transport proteins (Fig. 8). Downstream of *pelB* the gene *lpxA*, coding for UDP-N-acetylglucosamine acetyltransferase involved in lipid A biosynthesis, could be identified. These nucleotide sequences of *cydC, cydD* and *lpxA* (accession number: NC_007481) are located at position 2136039-2139540 of the *P. haloplanktis* TAC125 chromosome I.

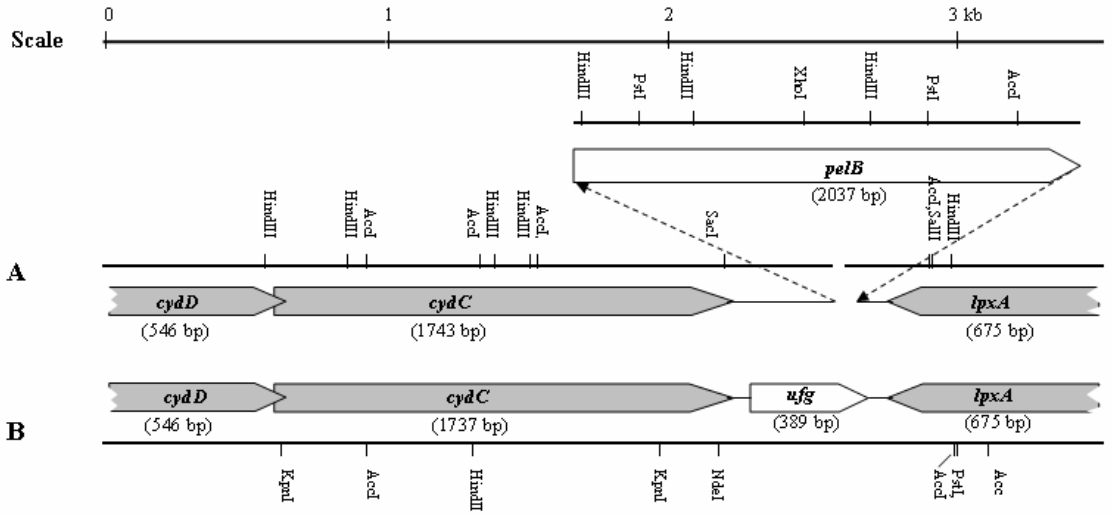


**Fig. 8.** Localization and molecular organization of the *pelB* gene on the genetic map of strain *P. haloplanktis* ANT/505 (A) compared to strain *P. haloplanktis* TAC125 (B). Shown are relevant restriction sites of the sequenced region. Amino acid sequences of ANT/505 with strong homology to regions of the TAC125 genome are highlighted in grey (*cydD* and *cydC*: cysteine transport (export) protein, *lpxA*: lipid A biosynthesis, UDP-N-acetylglucosamine acetyltransferase), *ufg*: unknown functional gene. The nucleotide sequence shown for TAC125 corresponds to the genome sequence region at position 2136039- 2139540 of chromosome I (Genbank accession number: NC_007481). The scale is given in kilobase pairs (Scale).

## Discussion

Based on the fact that the psychrophilic bacterium *P. haloplanktis* does not have the chance to find pectin so frequently in its preferred marine environment a strict regulation of its pectinolytic genes would be advantageous. However, the reference strain *P. haloplanktis* TAC125 does not express any pectinolytic activity. Besides, so far the strain *P. haloplanktis* ANT/505 is the only known marine bacterium expressing such enzymatic activity. Considering this, it could be speculated that the genes *pelA* and *pelB* have been acquired by this bacterium by horizontal gene transfer. Thus, from this point of view a regulation of these genes could not be postulated in the beginning. However, the data of this study clearly show that the transcription of the pectinolytic genes is regulated in the *P. haloplanktis* strain ANT/505. Most of the bacterial pectinases are regulated by different mechanisms, but the main inducer is frequently the substrate pectin. In this study it is shown that the *pelA* and *pelB* genes of *P. haloplanktis* are only significantly induced if pectin is available in the medium.

It is interesting to note that the *pelA* and *pelB* mRNAs showed a comparable expression. This indicates that both genes are regulated by the same mechanism. By means of primer extension the promoter regions of both cistrons were detected. A sequence analysis did not give any hint that the regulation of the *pelA* and *pelB* genes of *P. haloplanktis* requires a common regulator. One of the major regulators, determining the pectin dependent regulation of many pectinolytic genes in other bacteria, is the KdgR repressor (James and Hugouvieux-Cotte-Pattat 1996), Thomson et al. 1999). In other bacteria also binding sequences of the transcriptional repressors PecS (Reverchon, Nasser et al. 1994) and PecT (Surgey, Robert-Baudouy et al. 1996) play an important role in the regulation of pectinolysis. However, no typical consensus sequences or palindrome structures for these known regulators could be identified in the promoter regions of *pelA* and *pelB* of *P. haloplanktis* strain ANT/505 in this study. It has been reported that other proteins involved in global regulations, such as CRP or HNS, can bind to the regulatory regions of the pectinase genes and affect their transcription (Hugouvieux-Cotte-Pattat, Condemine et al. 1996). In this respect it is interesting to note that there seems to be no catabolite repression in *P. haloplanktis*. This marine bacterium is lacking the cAMP-CAP complex that regulates carbon availability in other bacteria (Medigue, Krin et al. 2005). Furthermore, apparently *P. haloplanktis* is not able to metabolize glucose, since this bacterium does not possess a phosphoenolpyruvate-dependent phosphotransferase system, which is usually required for the transport of this carbon and energy source.

The sequence analysis of the genes upstream and downstream of *pelB* revealed that this gene is located on chromosome I of *P. haloplanktis*. Several attempts to amplify the chromosomal DNA fragment located between the *pelB* and the *pelA* gene by long-range PCR failed. This indicates that *pelA* and *pelB* are either located separately from each other on chromosome I or that *pelA* is located on chromosome II. This also suggests that if these enzymatic activities were acquired by *P. haloplanktis* ANT/505 by horizontal gene transfer the genes were incorporated into the genome by two independent recombination events.

Results of this study demonstrate that the pectinase genes are expressed from independent cistrons, which are not clustered but located at distant positions on chromosome I of the *P. haloplanktis* genome. Further investigations are needed to elucidate the specific regulation of these pectinolytic genes uncommon for a marine bacterium. For this purpose, the cells of *P. haloplanktis* should be accessible by molecular biological methods. In this respect first successful genetic manipulations of *P. haloplanktis* cells described by Tutino et al. (2001) and Papa et al. (2006) are promising.

# Chapter V

# Summary and general discussion

## 1. Cold-adapted marine microorganisms and their enzymes

The marine phytoplancton at permanent cold environments produces a variety of polymers such as chitin, cellulose, glucan or starch. Although relatively little data are available about the hydrolysis of such compounds under permanent low temperature conditions, it is obvious that these structural or storage polymers can be degraded by marine psychrophilic microorganisms. During this thesis microorganisms isolated from ice, sea water or sediment from the Arctic or Antarctic Ocean have been characterized according to their ability to degrade pectin. The results of a pre-screening for different enzymatic activities are summarized in Table 1. Most of the isolated bacteria showed a fast growth at 5°C. It is worth to emphasize that many of the bacteria listed in Table 1 represent new species.

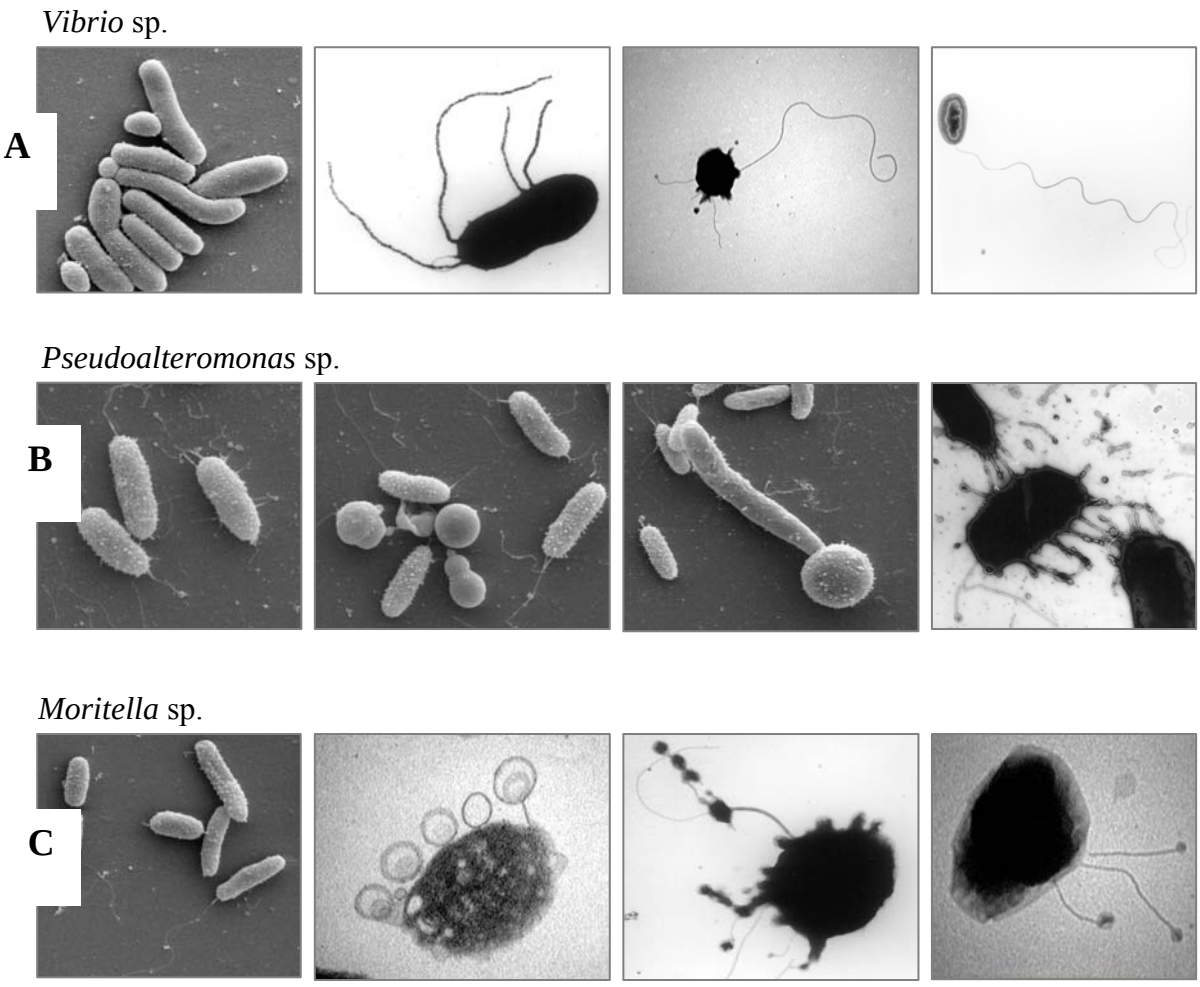


**Fig. 1** Transmission and raster electron micrographs of (A) *Vibrio* sp strain Fi:7, (B) *Pseudoalteromonas* sp. strain St10 and (C) *Moritella* sp. S62

| | Strain | Growth (day) | | | Protease activity | | Amylase activity | | Glucanase activity | | Chitinase activity | | Pectinase activity | | Taxonomical Affiliation* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5°C | 10°C | RT | pH | T (°C) | pH | T (°C) | pH | T (°C) | pH | T (°C) | pH | T (°C) | |
| 1 | 61 | 2 | 1 | 1 | + | + | - | - | + | + | nd | nd | nd | nd | *Vibrio* sp. |
| **2** | **505** | **2** | **1** | **1** | 7 | 37 | 7.5 | 25 | 8.0 | 40 | nd | nd | **10** | **30** | *P. haloplanktis* |
| 3 | 572 | 5 | 4 | 3 | + | + | 6 | 32 | 7.5 | nd | nd | nd | nd | nd | *Flavobacterium* sp. |
| 4 | 157 | 2 | no | no | nd | nd | - | - | 8.5 | 25 | nd | nd | nd | nd | |
| 5 | 72 | 6 | 4 | 1 | - | - | 6.0 | 35 | + | + | nd | nd | - | - | |
| **6** | Fi:7 | **2** | **1** | **1** | nd | nd | nd | nd | nd | nd | **8** | **30** | nd | nd | *Vibrio* sp. |
| 7 | 116 | 2 | w | no | nd | nd | 6 | 20 | 6 | 32 | nd | nd | nd | nd | |
| 8 | 11a | 2 | 1 | 1 | 6.2 | 40 | - | - | 8.5 | 25 | nd | nd | - | - | |
| 9 | 4a | 2 | 1 | 1 | 6 | 40 | - | - | - | - | nd | nd | - | - | |
| 10 | St27 | 2 | 1 | 1 | 6 | 35 | - | - | 8.5 | 25 | nd | nd | - | - | |
| 11 | Z1-21 | 2 | 1 | 1 | 6 | 40 | - | - | 8 | 30 | nd | nd | - | - | |
| 12 | **St10** | 2 | 1 | 1 | + | + | - | - | 7 | 35 | nd | nd | - | - | *Pseudoalteromonas* sp. |
| 13 | **21** | 2 | w | no | - | - | 7.5 | 27 | 7 | 30 | nd | nd | - | - | *Colwellia* sp. |
| 14 | 4b | 2 | 1 | 1 | - | - | 6.2 | 25 | 6 | 35 | nd | nd | - | - | |
| 15 | P26 | 2 | w | no | - | - | 6 | 20 | - | - | nd | nd | - | - | *Flavobacterium* sp. |
| 16 | S62 | 2 | 1 | no | nd | nd | nd | nd | nd | nd | 7 | 20 | - | - | *Moritella* sp. |

**Tab. 1** Characterisation of selected psychrophilic bacteria isolated from ice, sea water or sediment from the Arctic or Antarctic ocean according to their ability to degrade starch, glucan, casein, chitin and pectin.
*Based on sequencing of their 16S rRNAs
Not growth (no) and weak growth (w).
Positive (+) or negative (-) with substrates on solid medium. (nd): not determinded.

Characterization of selected isolates by means of transmission electron or raster electron microscopy showed very pleomorphic structures throughout their growth (Fig. 1). Particularly the cells of *Pseudoalteromonas* sp. revealed intensive morphological conversions from rod-shaped to coccoid cells during the transition from the logarithmic to stationary growth phase. The decrease in size and change in shape from a rod to a coccus is a typical behavior of marine bacteria under nutrient limitation conditions (Novitsky and Morita 1976). It is supposed that this morphological change ensures a better ratio between the size and the surface of the cells allowing a more efficient uptake of the limited nutrients from the extracellular environment.

## 2. Isolation of a pectinolytic marine bacterium and cloning of two different pectate lyase genes

The major aim of this thesis was to identify cold-adapted pectinases. 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 was already in 1999 supposed by Cummings and Black that this polymer is also degraded by marine microorganisms. Reports on cold-adapted pectinases were however before this study restricted to psychrotrophic spoilage bacteria such as different strains of *Pseudomonas fluorescens* (Schlemmer, Ware et al. 1987). Thus, no cloning of genes of cold-adapted pectinases was reported before this thesis. Furthermore, no marine pectinolytic microorganism was described in the literature before.

The strain ANT/505, investigated in this study, is thus the first pectinolytic marine bacterium isolated from a permanent cold environment like the Antarctic ocean. The marine Antarctic isolate ANT/505 was isolated from over 500 strains from the Antarctic and Arctic ocean as the only bacterium which produced significant pectinolytic activities under the conditions used in this study. This reflects the negligible role of pectin as a carbon source in these permanent cold marine environments. The two pectate lyases described in this study represent the first pectinases found in marine microorganisms.

According to the 16S rRNA 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 like succinate, citrate or glycerol utilization. Similar differences in catabolic activities could be found between two subspecies of *P. haloplanktis* (Gauthier, Gauthier et al. 1995).

The sequencing of the pectinolytic clones of a ANT/505 gene library in *E. coli* and a sequence alignment with know protein sequences indicated that the encoded 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, the dependence of the enzymatic activities on an alkaline pH and the presence of $Ca^{2+}$ (Whitaker 1990). The activity of the pectate lyase domain of PelA was dependent on sodium, other monovalent cations like $K^+$ could not replace $Na^+$. A similar dependence on sodium was 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 ß-elimination (data not shown), which is the typical reaction of lyases (Collmer et al. 1988).

A significant homology of PelA and PelB of isolate 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) could be detected. 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 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* can not be functionally expressed in *E. coli* at 37 °C. A significant amylase activity could be only found at temperatures below 25 °C. During an 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 significant lower activity for both pectate lyases could be observed. Since PelB showed a half live 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 optimal in a temperature range from 30-37 °C, a compromise between the thermostability of such cold-adapted enzymes and the optimal temperature for the expression system has to be found.

## 3. Cloning and characterization of the methylesterase domain of the bifunctional pectinase PelA

The exceptionally big pectinolytic enzyme PelA, with a size of 200 kDa, is to our knowledge the biggest pectinase reported so far. The sequence analysis of the complete *pelA* gene of the psychrophilic marine bacterium *P. haloplanktis* ANT/505 indicated a bifunctional nature of the corresponding enzyme. Blast search analyses of the PelA amino acid sequence revealed immediately upstream of the pectate lyase domain a potential pectin methylesterase domain. Several conserved regions homologous to sequences of known pectin methylestrases could be detected. A sequence comparison to other protein sequences in the databases indicated an affiliation of the *pme* domain of PelA to the group 1 of pectin methylesterases. The middle part of the deduced amino acid sequence of PelA comprising about 447 amino acids showed 29% and 25% homology to pectin methylesterases from *E. carotovora* and from *E. chrisanthemi*, both of which had been suggested to belong to the group 1 of pectin methylesterases. With 51.7% the highest homology of the *pme* domain could be found to a potential pectin methylesterase from *Saccharophagus degradans* strain MD2-40.

Pectin-degrading enzymes have to be secreted out of the cells to reach their substrate. It was found that the pectate lyase PelB of *P. haloplanktis* ANT/505 was secreted into the extracellular medium (Truong et al. 2001). However, the PelA activity could only be located in the periplasm of *P. haloplanktis*. The sequence analysis of the N-terminus of PelA revealed a potential signal peptide with a length of 22 amino acids. In addition two potential membrane spanning domains could be detected in the peptide sequence regions 686:701 and 891:906 of PelA, indicating an incorporation of this enzyme into the cellular membrane.

By means of an enzymatic assay a clear methylesterase activity of PelA and the *pme* domain could be shown. While many pectinases have been found in microorganisms, such a bifunctional enzyme seems not to be that common (Keen and Tamaki 1986; Nasser, Chalet et al. 1990). Recently, a bifunctional pectinolytic enzyme with separate pectate lyase and pectin methylesterase domains from an alkaliphilic *Bacillus* have been described (Kobayashi, Sawada et al. 2003). Furthermore, in a recent study Solbak et al. (2005) used a high throughput screening assay with complex environmental metagenome DNA samples and could discover more than 40 novel microbial pectate lyases. It was shown that many of these pectate lyases were modular in structure and contained not only a pectate lyase catalytic domain belonging either to the polysaccharide lyase families 1 or 10, but had also methylesterase domains and a variety of other modules such as N- or C-terminal carbohydrate

binding modules. Several bifunctional enzymes containing a pectin methylesterase domain and a pectate lyase domain from either polysaccharide lyase family 1 or 10 could be discovered in this study. Some of the pectinolytic enzymes discovered by Solbak et al. (2005) also contained fibronectin type 3-like modules. Such a domain could also be detected in the N-terminal region of PelA. Although these domains are common among bacterial extracellular glycohydrolases their function has not yet been fully elucidated (Kataeva et al 2002).

### 4. Regulation of *pelA* and *pelB* expression

Based on the fact that the psychrophilic bacterium *P. haloplanktis* does not have the chance to find pectin so frequently in its preferred marine environment a strict regulation of its pectinolytic genes would be advantageous. However, the reference strain *P. haloplanktis* TAC125 does not express any pectinolytic activity. Besides, so far the strain *P. haloplanktis* ANT/505 is the only known marine bacterium expressing such enzymatic activity. Considering this, it could be speculated that the genes *pelA* and *pelB* have been acquired by this bacterium by horizontal gene transfer. Thus, from this point of view a regulation of these genes could not be postulated in the beginning. However, the data of this study clearly show that the transcription of the pectinolytic genes is regulated in the *P. haloplanktis* strain ANT/505. Most of the bacterial pectinases are regulated by different mechanisms, but the main inducer is frequently the substrate pectin. In this study it is shown that the *pelA* and *pelB* genes of *P. haloplanktis* are only significantly induced if pectin is available in the medium.
It is interesting to note that the *pelA* and *pelB* mRNAs showed a comparable expression. This indicates that both genes are regulated by the same mechanism. By means of primer extension the promoter regions of both cistrons were detected. A sequence analysis did not give any hint that the regulation of the *pelA* and *pelB* genes of *P. haloplanktis* requires a common regulator. One of the major regulators, determining the pectin dependent regulation of many pectinolytic genes in other bacteria, is the KdgR repressor (James and Hugouvieux-Cotte-Pattat 1996; Thomson, Nasser et al. 1999). In other bacteria also binding sequences of the transcriptional repressors PecS (Reverchon et al. 1994) and PecT (Surgey, Robert-Baudouy et al. 1996) play an important role in the regulation of pectinolysis. However, no typical consensus sequences or palindrome structures for these known regulators could be identified in the promoter regions of *pelA* and *pelB* of *P. haloplanktis* strain ANT/505 in this study. It has been reported that other proteins involved in global regulations, such as CRP or HNS, can bind to the

regulatory regions of the pectinase genes and affect their transcription (Hugouvieux-Cotte-Pattat, Condemine et al. 1996). In this respect it is interesting to note that there seems to be no catabolite repression in *P. haloplanktis*. This marine bacterium is lacking the cAMP-CAP complex that regulates carbon availability in other bacteria (Medigue, Krin et al. 2005). Furthermore, apparently *P. haloplanktis* is not able to metabolize glucose, since this bacterium does not possess a phosphoenolpyruvate-dependent phosphotransferase system, which is usually required for the transport of this carbon and energy source.

The sequence analysis of the genes upstream and downstream of *pelB* revealed that this gene is located on chromosome II of *P. haloplanktis*. Several attempts to amplify the chromosomal DNA fragment located between the *pelB* and the *pelA* gene by long-range PCR failed. This indicates that *pelA* and *pelB* are either located separately from each other on chromosome II or that *pelA* is located on chromosome I. This also suggests that if these enzymatic activities were acquired by *P. haloplanktis* ANT/505 by horizontal gene transfer the genes were incorporated into the genome by two independent recombination events.

Results of this study demonstrate that the pectinase genes are expressed from independent cistrons, which are not clustered but located at distant positions on chromosome II of the *P. haloplanktis* genome. Further investigations are needed to elucidate the specific regulation of these pectinolytic genes uncommon for a marine bacterium. For this purpose, the cells of *P. haloplanktis* should be accessible by molecular biological methods. In this respect first successful genetic manipulations of *P. haloplanktis* cells described by Tutino et al. (2001) and Papa et al. (2006) are promising.

# References


Aghajari, N., G. Feller, et al. (1998). "Crystal structures of the psychrophilic alpha-amylase from Alteromonas haloplanctis in its native form and complexed with an inhibitor." Protein Sci **7**(3): 564-72.

Aghajari, N., G. Feller, et al. (1998). "Structures of the psychrophilic Alteromonas haloplanctis alpha-amylase give insights into cold adaptation at a molecular level." Structure **6**(12): 1503-16.

Aghajari, N., F. Van Petegem, et al. (2003). "Crystal structures of a psychrophilic metalloprotease reveal new insights into catalysis by cold-adapted proteases." Proteins **50**(4): 636-47.

Ahrens, R. (1968). "Taxonomische Untersuchungen an sternbildenden Agrobacterium Arten aus der westlichen Ostsee." Kiel Meeresforsch **24**: 147-173.

Albersheim, P. and U. Kilias (1962). "Archives of Biochemistry and Biophysics." **97**: 107.

Alberts, B., D. Bray, et al. (1986). "Molekularbiologie der Zelle." 1241.

Batra, S. K. (1985). in Handbook of Fiber Science and Technology (Lewin, M., and Pearce, E. M., eds) Marcel Decker, New York: 727-807.

Bauer, D. W. and A. Collmer (1997). "Molecular cloning, characterization, and mutagenesis of a pel gene from Pseudomonas syringae pv. lachyrmans encoding a member of the Erwinia chrysanthemi pelADE family of pectate lyases." Mol Plant Microbe Interact **10**(3): 369-79.

Bell, G. S., R. J. Russell, et al. (2002). "Stepwise adaptations of citrate synthase to survival at life's extremes. From psychrophile to hyperthermophile." Eur J Biochem **269**(24): 6250-60.

Berger, F., N. Morellet, et al. (1996). "Cold shock and cold acclimation proteins in the psychrotrophic bacterium Arthrobacter globiformis SI55." J Bacteriol **178**(11): 2999-3007.

Berger, F., P. Normand, et al. (1997). "capA, a cspA-like gene that encodes a cold acclimation protein in the psychrotrophic bacterium Arthrobacter globiformis SI55." J Bacteriol **179**(18): 5670-6.

Bernfeld, P. (1955). "Amylases a and b. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic, New York." 149-155.

Borriss, M., T. Lombardot, et al. (2006). "Genome and proteome characterization of the psychrophilic Flavobacterium bacteriophage 11b." Extremophiles.

Burkholder, P. (1963). " Some nutritional relationships among microbes of the sea sediments and water. In: Symposium on marine microbiology. Oppenheimer H. (ed)." 133-155.

Carpita, N. C. and D. M. Gibeaut (1993). "Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth." Plant J **3**: 1-30.

Castelien, J. M. and a. l. et (1976). Lebensmittel-Wissencshaft und technolgie **9**: 277.

Chong, S. C., Y. Liu, et al. (2002). "Methanogenium marinum sp. nov., a H2-using methanogen from Skan Bay, Alaska, and kinetics of H2 utilization." Antonie Van Leeuwenhoek **81**(1-4): 263-70.

Collmer, A. and a. l. et (1988). "Assay methods for pectic enzymes. In: Wood WA, Kellog ST. (eds), Methods in enzymology. Academic SanDiego." 329-399.

Condemine, G. and J. Robert-Baudouy (1991). "Analysis of an Erwinia chrysanthemi gene cluster involved in pectin degradation." Mol Microbiol **5**(9): 2191-202.

Cummings, S. P. and G. W. Black (1999). "Polymer hydrolysis in a cold climate." Extremophiles **3**(2): 81-7.
D'Amico, S., T. Collins, et al. (2006). "Psychrophilic microorganisms: challenges for life." EMBO Rep **7**(4): 385-9.
de Vries, R. P. and J. Visser (2001). "Aspergillus enzymes involved in degradation of plant cell wall polysaccharides." Microbiol Mol Biol Rev **65**(4): 497-522, table of contents.
Dey, P. and K. Brinson (1984). " Plant cell walls." Adv.carbonhyd. Chem. Biochem **42**: 265-382.
Eastman, J. T. (1993). "Antarctic fish biology, evolution in a unique environment." Academic Press, San Diego.
Feller, G. and C. Gerday (1997). "Psychrophilic enzymes: molecular basis of cold adaptation." Cell Mol Life Sci **53**(10): 830-41.
Feller, G. and C. Gerday (2003). "Psychrophilic enzymes: hot topics in cold adaptation." Nat Rev Microbiol **1**(3): 200-8.
Feller, G., O. Le Bussy, et al. (1998). " Expression of psychrophilic genes in mesophilic hosts: assessment of the folding state of a recombinant alpha-amylase." Appl Environ Microbiol **64**: 1163-1165.
Feller, G., F. Payan, et al. (1994). "Stability and structural analysis of alpha-amylase from the antarctic psychrophile Alteromonas haloplanctis A23." Eur J Biochem **222**(2): 441-7.
Finkelman, M. J. and J. E. Zajic (1978). "Developments in Industrial Microbiology." 459.
Franzmann, P. D., Y. Liu, et al. (1997). "Methanogenium frigidum sp. nov., a psychrophilic, H2-using methanogen from Ace Lake, Antarctica." Int J Syst Bacteriol **47**(4): 1068-72.
Franzmann, P. D., N. Stringer, et al. (1992). "A methanogenic archaeon from Ace Lake, Antarctica: Methanococcoides burtonii sp. nov. System." Appl. Microbiol. Biotechnol **15**: 573–581.
Gauthier, G., M. Gauthier, et al. (1995). "Phylogenetic analysis of the genera Alteromonas, Shewanella, and Moritella using genes coding for small-subunit rRNA sequences and division of the genus Alteromonas into two genera, Alteromonas (emended) and Pseudoalteromonas gen. nov., and proposal of twelve new species combinations." Int J Syst Bacteriol **45**: 755-761.
Gerday, C., M. Aittaleb, et al. (2000). "Cold-adapted enzymes: from fundamentals to biotechnology." Trends Biotechnol **18**: 103-107.
Godfrey, T. and S. West (1996). " Industrial enzymology, 2nd edition, chapter 2.13.1." 236.
Gounot, A. M. (1999). "Microbial life in permanently cold soils. In: Margesin, R. and Schinner, F. (Eds) Cold-adapted Organisms, Ecology, Physiology, enzymology and Molecular Biology." Spriger: 4-15.
Hanahan, D. (1983). "Techniques of transformation in Escherichia coli. In: Glover DM (eds) DNA cloning 1." IRL Press, Oxford: 109-145.
Hebraud, M., E. Dubois, et al. (1994). "Effect of growth temperatures on the protein levels in a psychrotrophic bacterium, Pseudomonas fragi." J Bacteriol **176**(13): 4017-24.
Hébraud, M., P. Potier, et al. (2000). in Cold Shock, Response and Adaptation (eds Inouye, M. & Yamanaka, K.): 41−60.
Helmke, E. and H. Weyland (1984). "Rhodococcus marinonascens sp.nov., an actinomycete from the sea." Int J Syst Bacteriol **34**: 127-138.
Ho, M. C., M. P. Whitehead, et al. (1995). "Sequence analysis of the Aspergillus nidulans pectate lyase pelA gene and evidence for binding of promoter regions to CREA, a regulator of carbon catabolite repression." Curr. Genet **27**: 142-149.
Hohman, R. W. (1975). "Optimum temperatures and temperature ranges for growth of snow algae." Arctic Alpine Res **7**: 13-24.

Holland, L. Z., M. McFall-Ngai, et al. (1997). "Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus Sphyraena) from different thermal environments: differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site." Biochemistry **36**(11): 3207-15.

Homma, M., D. J. DeRosier, et al. (1990). "Flagellar hook and hook-associated proteins of Salmonella typhimurium and their relationship to other axial components of the flagellum." J. Mol. Biol. **213**(4): 819-832.

Hugouvieux-Cotte-Pattat, N., G. Condemine, et al. (1996). "Regulation of pectinolysis in Erwinia chrysanthemi." Annu Rev Microbiol **50**: 213-57.

Humphreys, D., N. Weir, et al. (1995). "Human protein disulfide isomerase functionally complements a dsbA mutation and enhances the yield of pectate lyase C in Escherichia coli." J Biol Chem **270**: 28210-28215.

James, V. and N. Hugouvieux-Cotte-Pattat (1996). "Regulatory systems modulating the transcription of the pectinase genes of Erwinia chrysanthemi are conserved in Escherichia coli." Microbiology **142 (Pt 9)**: 2613-9.

Jenkins, J., O. Mayans, et al. (2001). "Three-dimensional structure of Erwinia chrysanthemi pectin methylesterase reveals a novel esterase active site." J Mol Biol **305**(4): 951-60.

Jia, Z., C. I. DeLuca, et al. (1996). "Structural basis for the binding of a globular antifreeze protein to ice." Nature **384**(6606): 285-8.

Jones, L. P., R. Y. Morita, et al. (1979). " Fructose-1,6-bisphosphate aldohydrolase from Vibrio marinus, a psychrophic marine bacterium." Zeitschrift fur Allgemeine Mikrobiologie **19**: 97-106.

Jürgen, B., S. Tobisch, et al. (2005). "Global expression profiling of B. subtilis cells during industrial-close fed-batch fermentations with different nitrogen sources." Biotechnol Bioeng **92**: 277-98.

Kataeva, I. A., R. D. Seidel, 3rd, et al. (2002). "The fibronectin type 3-like repeat from the Clostridium thermocellum cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface." Appl Environ Microbiol **68**(9): 4292-300.

Keegstra, K. a. e. and a. el. (1973). "The structure of plant cell wall, III: a model of the walls of suspension-cultured sycamore cells based on the interconnection of the marcromolecular components." Plant physiol. **51**: 188-196.

Keen, N. T. and S. Tamaki (1986). "Structure of two pectate lyase genes from Erwinia chrysanthemi EC1 and their high-level expression in Escherichia coli." J. Bacteriol **168**: 595-606.

Kobayashi, T., K. Sawada, et al. (2003). "Bifunctional pectinolytic enzyme with separate pectate lyase and pectin methylesterase domains from an alkaliphilic Bacillus." World Journal of Microbiology & Biotechnology **19**: 269-277.

Kozianowski, G., F. Canganella, et al. (1997). " Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, Thermoanaerobacter italicus sp. nov." Extremophiles **1**: 171-182.

Laurent, P., L. Buchon, et al. (2000). "Production of pectate lyases and cellulases by Chryseomonas luteola strain MFCL0 depends on the growth temperature and the nature of the culture medium: evidence for two critical temperatures." Appl Environ Microbiol **66**:1538-1543.

Lee, R. E. and D. L. Denlinger (1991). "Insects at low temperatures."

Loppes, R., N. Devos, et al. (1996). "Effect of temperature on two enzymes from a psychrophilic Chloromonas (Chlorophyta)." J. Phycol **32**: 276-278.

Mac Cann, M. C. and K. Roberts (1994). "Plant cell walls: murals and mosaics." Agro-Food industry Hi-Tech: 43-46.

Macmillian, J. D. and a. l. et (1964). "Biochemistry." **3**: 564.

Macmillian, J. D. and a. l. et (1966). "Methods in enzymology." **8**: 632.

Medigue, C., E. Krin, et al. (2005). "Coping with cold: the genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125." Genome Res **15**(10): 1325-35.
Mitchell, P. and a. l. et (1985). "Propaties of lactte dehydroenase in psychrophilic marine bacterium." Appl Environ Microbiol **49**: 1332-1334.
Monro, J., D. Penny, et al. (1976). " Review: the organisation and growth of primary cell walls of lupin hypocotyls." Phytochemistry **15**: Phytochemistry.
Morita, R. (1975). "Psychrophilic bacteria." Bacteriol Rev **39**: 144-169.
Narinx, E. and a. l. et (1997). "Subtilisin from psychrophilic Antartic bacteria: characterization and site directed mutagenesis of residues possibly involved in the adaptation to cold." Protein Eng **11**: 1171-1279.
Nasser, W., F. Chalet, et al. (1990). " Purification and characterization of extracellular pectate lyase from Bacillus subtilis." Biochimie **72**: 689-695.
Nasser, W., S. Reverchon, et al. (1994). "Specific interactions of Erwinia chrysanthemi KdgR repressor with different operators of genes involved in pectinolysis." J Mol Biol **236**(2): 427-40.
Nasser, W., J. Robert-Baudouy, et al. (1997). "Antagonistic effect of CRP and KdgR in the transcription control of the Erwinia chrysanthemi pectinolysis genes." Mol Microbiol **26**(5): 1071-82.
Nichols, D., P. Nichols, et al. (1995). in Sci Progr Oxf. **78**: 311.
Nickel, M., G. Homuth, et al. (2004). "Cold induction of the Bacillus subtilis bkd operon is mediated by increased mRNA stability." Mol Genet Genomics **272**(1): 98-107.
Novitsky, J. A. and R. Y. Morita (1976). "Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio." Appl Environ Microbiol **32**(4): 617-22.
Ohgiya, S. (1999). "Biotechnology of enzymes from cold-adapted microorganisms. In: Margesin, R. and Schinner F. (Eds.) Biotechnology of enzymes from cold-adapted microorganisms." Spriger: 17-34.
Okamoto, K. (1963). "Agricultural and Biological chemistry." **27**: 596.
Okamoto, K. (1964). "Berichte des Ohara institut für Landwirtschaftliche biologie, Okayama University." **12**: 115.
Papa, R., V. Rippa, et al. (2006). "An effective cold inducible expression system developed in Pseudoalteromonas haloplanktis TAC125." J Biotechnol.
Pilling, J., L. Willmitzer, et al. (2000). "Expression of a Petunia inflata pectin methyl esterase in Solanum tuberosum L. enhances stem elongation and modifies cation distribution." Planta **210**(3): 391-9.
Pissavin, C., J. Robert-Baudouy, et al. (1996). "Regulation of pelZ, a gene of the pelB-pelC cluster encoding a new pectate lyase of Erwinia chrysanthemi 3937." J Bacteriol **178**: 7187-7196.
Raymond, J. A., P. Wilson, et al. (1989). "Inhibition of growth of nonbasal planes in ice by fish antifreezes." Proc Natl Acad Sci U S A **86**(3): 881-5.
Redenbach, M., H. M. Kieser, et al. (1996). "A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb Streptomyces coelicolor A3(2) chromosome." Mol Microbiol **21**: 77-96.
Reverchon, S., W. Nasser, et al. (1991). "Characterization of kdgR, a gene of Erwinia chrysanthemi that regulates pectin degradation." Mol Microbiol **5**(9): 2203-16.
Reverchon, S., W. Nasser, et al. (1994). "pecS: a locus controlling pectinase, cellulase and blue pigment production in Erwinia chrysanthemi." Mol Microbiol **11**(6): 1127-39.
Roland, J. C. (1980). "Polymereses vegetaux. Ultrastructure et texture des polysaccharides dans la paroides cellules vegetales. In: Moties, B. (Ed.) Enzyme d´interet Industriel. Gauthier - Villars, Paris." 46-65.

Rosenberg, M. and D. Court (1979). "Regulatory sequences involved in the promotion and termination of RNA transcription." Annu Rev Genet **13**: 319-53.
Russell, N. J. (1990). "Cold adaptation of microorganisms." Phil. Trans. R. Soc. London **B326**: 595–611.
Russell, N. J. (1992). "Physiology and molecular biology of psychrophilic microorganisms." In: Molecular Biology and Biotechnology of Extremophiles, Herbert R. A. and Sharp R. J. (eds). Blackie, London: 203–224.
Russell, N. J. (1998). "Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications." Adv Biochem Eng Biotechnol **61**: 1-21.
Russell, N. J. (2000). "Toward a molecular understanding of cold activity of enzymes from psychrophiles." Extremophiles **4**(2): 83-90.
Russell, R. J., U. Gerike, et al. (1998). "Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium." Structure **6**(3): 351-61.
Sambrook, J., E. F.Fritsch, et al. (2001). "Molecular cloning: a laboratory manual, 3nd ed. Cold Spring Harbor Laboratory, Cold spring Harbor. N.Y."
Sambrook, J., E. F. Fritsch, et al. (1989). "Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold spring Harbor. N.Y."
Schlemmer, A. F., C. F. Ware, et al. (1987). "Purification and characterization of a pectin lyase produced by Pseudomonas fluorescens W51." J Bacteriol **169**(10): 4493-8.
Schmoll, T., H. Hoschutzky, et al. (1989). "Analysis of genes coding for the sialic acid-binding adhesin an tow other minor fimbrial subunits of the S-fimbrial adhesin determinant of E. coli." Mol Microbiol **3**: 1735-1744.
Schols, H. A., M. A. Posthumus, et al. (1990). "structural features of hairy region of pectin isolated from apple juice producted by the liquefation process." carbonhydr Res **206**: 117-129.
Schweder, T., E. Kruger, et al. (1999). "Monitoring of genes that respond to process-related stress in large-scale bioprocesses." Biotechnol Bioeng **65**(2): 151-9.
Seagull, R. W., V. Oliveri, et al. (2000). J. Cotton Sci. **4**: 97-104.
Searle-van Leeuwen, M. J. F., J.-P. Vincken, et al. (1996). "Acetyl esterases of Aspergillus niger: purification and mode of action on pectins." In: Visser J, Voragen A G J., editors. Pectins and pectinases. Vol. 14. Amsterdam, The Netherlands: Elsevier Science: 793-798.
Shevchik, V. E. and N. Hugouvieux-Cotte-Pattat (1997). "Identification of a bacterial pectin acetyl esterase in Erwinia chrysanthemi 3937." Mol Microbiol **24**(6): 1285-301.
Shevchik, V. E., J. Robert-Baudouy, et al. (1997). "Pectate lyase PelI of Erwinia chrysanthemi 3937 belongs to a new family." J. Bacteriol **179**: 7321-7330.
Simankova, M. V., S. N. Parshina, et al. (2001). "Methanosarcina lacustris sp. nov., a new psychrotolerant methanogenic archaeon from anoxic lake sediments." Syst Appl Microbiol **24**(3): 362-7.
Singh, N., M. M. Kendall, et al. (2005). "Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in Skan Bay, Alaska: description of Methanococcoides alaskense sp. nov., and emended description of Methanosarcina baltica." Int J Syst Evol Microbiol **55**(Pt 6): 2531-8.
Smalas, A. O., H. K. Leiros, et al. (2000). "Cold adapted enzymes." Biotechnol Annu Rev **6**: 1-57.
Solbak, A. I., T. H. Richardson, et al. (2005). "Discovery of pectin-degrading enzymes and directed evolution of a novel pectate lyase for processing cotton fabric." J Biol Chem **280**(10): 9431-8.
Somogyi, M. (1952). Journal of Biological Chemistry **195**: 19.
Soriano, M., A. Blanco, et al. (2000). "An unusual pectate lyase from a Bacillus sp. with high activity on pectin: cloning and characterization." Microbiology **146 (Pt 1)**: 89-95.

Surgey, N., J. Robert-Baudouy, et al. (1996). "The Erwinia chrysanthemi pecT gene regulates pectinase gene expression." J Bacteriol **178**(6): 1593-9.
Taguchi, S., A. Ozaki, et al. (1998). "Engineering of a cold-adapted protease by sequential random mutagenesis and a screening system." Appl Environ Microbiol **64**(2): 492-5.
Tamaki, S. J., S. Gold, et al. (1988). "Structure and organization of the pel genes from Erwinia chrysanthemi EC16." J Bacteriol **170**(8): 3468-78.
Tardy, F., W. Nasser, et al. (1997). "Comparative analysis of the five major Erwinia chrysanthemi pectate lyases: enzyme characteristics and potential inhibitors." J Bacteriol **179**(8): 2503-11.
Thomson, N. R., W. Nasser, et al. (1999). "Erwinia carotovora has two KdgR-like proteins belonging to the IciR family of transcriptional regulators: identification and characterization of the RexZ activator and the KdgR repressor of pathogenesis." Microbiology **145 (Pt 7)**: 1531-45.
Tierny, Y., C. G. Hounsa, et al. (1999). "Effects of a recombinant gene product and growth conditions on plasmid stability in pectinolytic Escherichia coli cells." Microbios **97**(386): 39-53.
Truong, L. V., H. Tuyen, et al. (2001). "Cloning of two pectate lyase genes from the marine Antarctic bacterium Pseudoalteromonas haloplanktis strain ANT/505 and characterization of the enzymes." Extremophiles **5**(1): 35-44.
Tsujibo, H., T. Ohtsuki, et al. (1997). "Cloning and sequence analysis of genes encoding xylanases and acetyl xylan esterase from Streptomyces thermoviolaceus OPC-520." Appl Environ Microbiol **63**(2): 661-4.
Tsuruta, H., B. Mikami, et al. (2005). "Crystal structure of cold-active protein-tyrosine phosphatase from a psychrophile, Shewanella sp." J Biochem (Tokyo) **137**(1): 69-77.
Tutino, M. L., A. Duilio, et al. (2001). "A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature." Extremophiles **5**(4): 257-64.
Volker, U., H. Mach, et al. (1992). "Stress proteins and cross-protection by heat shock and salt stress in Bacillus subtilis." J Gen Microbiol **138**(10): 2125-35.
von Klein, D., H. Arab, et al. (2002). "Methanosarcina baltica, sp. nov., a novel methanogen isolated from the Gotland Deep of the Baltic Sea." Extremophiles **6**(2): 103-10.
Wetzstein, M., U. Volker, et al. (1992). "Cloning, sequencing, and molecular analysis of the dnaK locus from Bacillus subtilis." J Bacteriol **174**(10): 3300-10.
Weyland, H., H.-J. Rüger, et al. (1970). "Zur Isolierung und Identifizierung mariner Bakterien, Veröff. Inst. Meeresf. Bremerh." **12**: 269-296.
Whitaker, J. R. (1990). "Microbial pectolytic enzymes. In: William M. Fogerty and Gatherine T. Kelly (eds)." Microbial enzymes and biotechnology, 2nd edition.
Wojciechowski, C. L. and R. Fall (1996). "A continuous fluorometric assay for pectin methylesterase." Anal Biochem **237**(1): 103-8.
Yoder, M. D., N. T. Keen, et al. (1993). "New domain motif: the structure of pectate lyase C, a secreted plant virulence factor." Science **260**(5113): 1503-7.

## List of publications

**1. Truong, L. V.**; Tuyen, H.; Helmke, E.; Binh, L. T. and Schweder, T. (2001). "Cloning of two pectate lyase genes from the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505 and characterization of the enzymes." Extremophiles 5(1): 35-44.

**2. Truong, L. V**. and Schweder, T. (2006)
"A bifunctional pectinolytic enzyme from the psychrophilic marine bacterium *Pseudoaltermonas haloplanktis* ANT/505". (In preparation)

**3. Truong, L. V**. and Schweder, T. (2006)
"Regulation of the pectinolytic genes of the psychrophilic marine bacterium *Pseudoalteromonas haloplanktis* ANT/505". (In preparation)

# CURRICULUM VITAE

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## Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Greifswald, den 21. 9. 2006 Unterschrift

Le Van Truong