Pectate lyase C from *Bacillus subtilis*: a novel endo-cleaving enzyme with activity on highly methylated pectin

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The gene *yvpA* from *Bacillus subtilis* was cloned and expressed in *Escherichia coli*. It encoded a pectate lyase of 221 amino acids that was denominated PelC. The heterologously expressed enzyme was purified by His-tag affinity chromatography and characterized. PelC depolymerized polygalacturonate and pectins of methyl esterification degree from 22% to 89%, exhibiting maximum activity on 22% esterified citrus pectin. It showed an absolute Ca$^{2+}$ requirement and the optimum temperature and pH were 65 °C and pH 10, respectively. The deduced amino acid sequence of PelC showed 53% identity to pectate lyase PeIA from *Paenibacillus barcinonensis*, which was also characterized. Similarly to PelC, purified PeIA showed activity on polygalacturonate and pectins with a high degree of methyl esterification. The two enzymes cleaved pectic polymers to a mixture of oligogalacturonates, indicating an endo mode of action. Analysis of activity on trigalacturonate showed that PelC cleaved it to galacturonic acid and unsaturated digalacturonate, whereas PeIA did not show activity on this substrate. PelC and PeIA showed high homology to a few recently identified pectate lyases of family 3 and form with them a cluster of small-sized pectate lyases from non-pathogenic micro-organisms.

INTRODUCTION

Pectin is a plant polysaccharide that contributes to the structure of plant tissues as a component of the middle lamella and primary cell wall. It consists of a backbone of D-galacturonic acid residues which are partially methyl esterified. The main chain of the polymer also contains rhamnose residues that can be highly substituted by arabinose and galactose side chains (Schols & Voragen, 2003). Pectin degradation requires the combined action of several enzymes, among which two main groups can be distinguished: pectinesterases, which remove methoxyl groups from pectin, and depolymerases (hydrolyases and lyases) that degrade the backbone chain. Lyases cleave glycosidic bonds by β-elimination, giving rise to unsaturated products. Among these enzymes, pectate lyases are specific for unmethylated polygalacturonate (pectate), although they can be active on pectins with a low degree of methyl esterification (Benen & Visser, 2003; Tardy *et al*., 1997). Pectate lyases are widely distributed among microbial plant pathogens, where they play an important role as virulence factors (Barras *et al*., 1994; Herron *et al*., 1997). They have also been found in saprophytic micro-organisms, including members of the genus *Bacillus* (Berenmeier *et al*., 2004; Hatada *et al*., 2000; Nasser *et al*., 1993) and in some thermophilic bacteria (Kluskins *et al*., 2003). Multiple sequence alignment of pectate lyases has been used to classify these enzymes into several subfamilies and to identify consensus sequences of some of them (Coutinho & Henrissat, 1999; Shevchik *et al*., 1997). The study of the three-dimensional folding of pectate lyase C from *Erwinia chrysanthemi* led to the finding of a novel protein structure, a parallel-right handed β-helix (Yoder *et al*., 1993), that is found in pectate lyases of families 1, 3 and 9 (Jenkins *et al*., 2004).

Pectin-degrading enzymes are widely used in the food industry for improving the yield and the clarification of fruit juices (Kashyap *et al*., 2001). Alkaline pectinases are being introduced in the textile industry to release fibres from flax stems, as an alternative to conventional retting (Hoondal *et al*., 2002). In these fields, finding new enzymes has special interest to improve the efficiency of the production systems.

In a previous study, pectate lyase PeIA from *Paenibacillus barcinonensis* was identified and characterized (Soriano *et al*., 2000). As a distinctive trait among known pectate lyases, the new enzyme showed high activity on both polygalacturonate and highly methylated pectins. Analysis of the PeIA amino acid sequence showed that the enzyme did not display homology to pectate lyases from saprophytic micro-organisms, while high homology was found to the hypothetical protein deduced from the *yvpA* gene from *Bacillus subtilis* (Kunst *et al*., 1997). In this article we describe the cloning of *yvpA*, and show that it encodes a pectin-degrading enzyme that has been denominated PeC. The new pectate lyase shares with PeIA a high activity on highly...
methylated pectins and defines, together with PeA, a cluster of family 3 pectate lyases from non-pathogenic microorganisms.

**METHODS**

**Bacterial strains and plasmids.** *Bacillus subtilis* 168 was obtained from the Spanish Type Culture Collection (CECT 461) and cultivated at 30 °C in nutrient broth. *Escherichia coli* 5K (Soriano et al., 2000) and *E. coli* BL21(DE3) (Studier & Moffatt, 1986) were cultivated at 37 °C in LB medium and used as recipient strains for recombinant plasmids. Plasmids pJF118HE (Fürste et al., 1986) and pET28α (Studier & Moffatt, 1986) were used as cloning vectors. *E. coli* plp22 has been previously described (Soriano et al., 2000). It harbours recombinant plasmid pET28α containing the pelA gene from *Paenibacillus barcinonensis* (Sánchez et al., 2005) cloned in pBR322.

**Nucleic acid manipulation.** Isolation of plasmids, restriction digestion of DNA, ligation of DNA and transformation of *E. coli* were carried out by standard methods. Chromosomal DNA from *B. subtilis* and *P. barcinonensis* was prepared as previously described (Soriano et al., 2000). PCR amplification and sequencing of DNA were done according to standard methodology.

The *yvpA* gene was PCR amplified with *Pfu* (Stratagene) with oligonucleotides 5′-ATCAGCGGAGAATGCAGTT-C3′ and 5′-CCGGAGCTCTTCATCAGA-3′. Those oligonucleotides are located 179 bp upstream and 141 bp downstream of the initiation and stop codons, respectively, of *yvpA* structural gene. The amplified fragment was ligated to plasmid pIF118HE previously digested with *Hin* II and *Nhe* I and transformed into *E. coli* 5K. The recombinant plasmid pET28α-yvpA obtained encoded PelC, devoid of pelA, and transformed into *E. coli* 5K. A similar approach was used. The oligonucleotides used for amplification were 5′-9-TTGCAGGCTGACAAGTG-3′ and 5′-CCGAGCTCTTCATCAGA-3′. Those oligonucleotides are located 179 bp upstream and 141 bp downstream of the initiation and stop codons, respectively, of *yvpA* structural gene. The amplified fragment was ligated to plasmid pIF118HE previously digested with *Sma* I, and introduced by transformation into *E. coli* 5K. The recombinant strain containing the *yvpA* gene was denominated *E. coli/plYPvA*.

Plasmids for overexpression of pectate lyases under the control of the T7 RNA polymerase system (Studier & Moffatt, 1986) were constructed as follows. *yvpA* was amplified with oligonucleotides 5′-TTGCAGGCTGACAAGTG-3′ and 5′-CCGGAGCTCTTCATCAGA-3′. Those oligonucleotides are located 179 bp upstream and 141 bp downstream of the initiation and stop codons, respectively, of *yvpA* structural gene. The amplified fragment was digested with *Nhe* I and *Hind* III, inserted between the *Nhe* I and *Hind* III sites of plasmid pET28α, and transformed into *E. coli* BL21(DE3). The recombinant plasmid pET28α-YvpA obtained encoded PeA, devoid of *pelA*, structural gene. The amplified fragment was digested with *Nhe* I and *Hind* III, inserted between the corresponding sites of pET28α. The recombinant plasmid pET28α-PeA encoded a mature PeA with an N-terminal Gly-Ser-His-Met extension, after removal of the His-tag.

**Purification of pectate lyases.** Purification of PelC and PeA was performed by His-tag affinity chromatography using NTA-Ni agarose resin (Qiagen) essentially as described by the pET28α supplier (Novagen). Recombinant clones *E. coli/plpET28α-YvpA* and *E. coli/plpET28α-PeA* were cultivated at 37 °C in 1 litre of kanamycin-supplemented LB to OD₆₀₀ 0.5–0.6. Then 1 mM IPTG was added and the cultures were further incubated for 150 min. Cells were collected, resuspended in 20 ml buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed in a French press. Cell extracts were clarified by centrifugation and His-tagged proteins were purified from the extracts. The His-tag of purified proteins was removed using the Thrombin Cleavage Capture Kit from Novagen.

**Enzyme assays.** Pectate lyase activity was assayed spectrophotometrically by measuring the formation of unsaturated products from pectic substrates at 232 nm (Collmer et al., 1988). The standard assay mixture contained 0-2% (w/v) substrate (Sigma) in a final volume of 3 ml 50 mM glycine buffer pH 10-0, containing 0-5 mM CaCl₂ for PeA activity determination or 2 mM CaCl₂ for PeC activity determination. Assay mixtures were incubated for 2-5 min at 50 °C and the increase of A₅₇₅ was measured. The substrates used were polygalacturonic acid and pectins of various degrees of methylation (22, 64 and 89% esterified citrus pectin, Sigma). Apple pectin (68% esterification, Sigma) was also tested as a substrate. One unit of enzymic activity was defined as the amount of enzyme that produces 1 μmol unsaturated product per minute under the assay conditions described. To study the effect of pH on activity, assays were performed at 65 °C (PelC) or 55 °C (PeA), replacing 50 mM sodium glycine buffer pH 10-0 by the following buffers: 50 mM sodium citrate pH 3-0–4-0, 50 mM sodium acetate pH 4-0–6-0, 50 mM sodium phosphate pH 6-0–8-0, 50 mM Tris/HCl pH 8-0–9-0, and 50 mM sodium glycine pH 9-0–12-0. The effect of temperature was studied at pH 10 in the temperature range 30–85 °C. For inhibition studies, 1 mM different ions were added to the assay mixture and activity was determined as indicated.

For stability assays, enzymes were incubated at 50 °C in buffers at pH 7-0 or 10-0 and samples were taken at regular intervals. Residual activity after thermal treatment was determined by the standard assay.

Kinetic parameters Vₘₐₓ and Kₘ were determined by fitting hyperbolic Michaelis–Menten curves with the program SigmaPlot version 8.0 (SPSS). Kinetic parameters of PeC and PeA on 22% methyl esterified pectin and polygalacturonic acid were determined with concentrations of substrates from 0.25 to 6 mg ml⁻¹, at 65 °C and 55 °C, respectively, in buffers at pH 10.

**Analytical procedures.** SDS-PAGE was performed in 13% (w/v) gels, essentially as described by Laemmli (1970). Samples were heat treated for 10 min at 45 °C in sample buffer before being applied to gels. After electrophoresis, gels were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in 100 mM glycine buffer pH 10-0, 1.5 mM CaCl₂ for 30 min, and overlaid with a 1% (w/v) agarose gel containing 1% (w/v) pectin in the same buffer as above. After 30 min incubation at 45 °C, gels were stained with 0.05% (w/v) ruthenium red (Sigma) for 10 min and washed with water until pectate lyase bands became visible.

Analysis of PeC and PeA reaction products was performed by TLC on silica gel plates (Kieselgel 60 F₂₅₄₅, Merck). The solvent used was n-butanol/water/acidic acid (5:3:2, by vol.). Oligosaccharides were detected by spraying the dried plates with a solution of 3% (w/v) phosphomolybdic acid and 10% (v/v) sulphuric acid in ethanol, and heating at 125 °C for 5 min. Galacturonic, digalacturonic and trigalacturonic acids (Sigma) were used as size markers.

**RESULTS**

**Cloning of the pectate lyase**

In a previous study a novel pectate lyase with high activity on both polygalacturonic acid and highly methylated pectin was cloned and characterized from *Paenibacillus barcinonensis* (Soriano et al., 2000). The deduced amino acid sequence of the enzyme, PeA, did not show homology to pectate lyases from saprophytic micro-organisms, but it displayed high homology to the protein deduced from *yvpA* gene from *Bacillus subtilis*, of unknown function (Kunst et al., 1997), indicating that it probably encoded a pectin-degrading enzyme. *yvpA* from *B. subtilis* was isolated by PCR
and cloned in E. coli. The recombinant clone obtained, E. coli/pJYvpA, produced haloes of substrate degradation when grown on polygalacturonic acid supplemented agar plates, showing that yvpA encoded a pectinase. Cell extracts from the recombinant clone showed lyase activity on polygalacturonic acid and pectin, while hydrolase activity on these substrates was undetectable. The results suggested that yvpA encoded a pectate lyase with similar substrate specificity to PelA.

Sequence analysis

The nucleotide sequence of the cloned gene was identical to that reported for yvpA (Kunst et al., 1997). It encodes a protein of 221 amino acids with predicted molecular mass and pI of 24281 Da and 8.85, respectively. The deduced amino acid sequence shows an N-terminal region of 27 amino acids with TTG as initiation codon. This start codon is frequently used in peptide (Nagarajan, 1993). The open reading frame starts with a sequence of GAGGA, similar to those recognized by the (Shine–Dalgarno) site. A putative promoter sequence, resembling that of a ribosome-binding site, was found upstream of the yvpA structural gene. An inverted repeat of 14 bp, that could act as a transcriptional terminator, was found in the 3′ region of the structural gene.

The deduced amino acid sequence of the cloned enzyme was compared to protein sequences in the Swissprot and EMBL databases. Homology was found to pectate lyases belonging to family PL3, while no homology was found to pectate lyases of other families (Fig. 1). The highest homology was found to pectate lyase from Bacillus sp. P-2850 (accession no. Q8L0R5), the protein deduced from yvpA gene from Bacillus licheniformis (accession no. Q65EF5), pectate lyase from Bacillus sp. KSM-P15 (Hatada et al., 2000) and PelA from P. barcinonesis (Soriano et al., 2000), which showed 72, 72, 53 and 53 % identity, respectively, to the cloned enzyme. High homology was also found to open reading frames deduced from genes SCO1110 from Streptomyces coelicolor (Bentley et al., 2002) and SAV6383 from Streptomyces avermitilis (Omurah et al., 2001), which showed 44 and 40 % identity to the cloned enzyme. Homology was also found to pectate lyases from phytopathogenic fungi and bacteria: P1 from Fusarium oxysporum (Huertas-González et al., 1999), Pell from Erwinia chrysanthemi (Shervisk et al., 1997), PelB from Fusarium solani (Guo et al., 1995) and Pel3 from Erwinia carotovora (Liu et al., 1994), which showed 43, 42, 37 and 35 % identity, respectively, to the cloned pectate lyase.

The cloned enzyme did not show homology to the two pectin-degrading enzymes, a pectate lyase (Nasser et al., 1993) and a pectin lyase (Sakamoto et al., 1996), characterized to date from B. subtilis. We named the cloned enzyme PelC and propose the encoding gene to be designated pelC instead of yvpA. The amino acid sequence of PelC contains three out of the four blocks of conserved residues proposed as signatures of pectate lyases from family 3 (Shevchik et al., 1997) (Fig. 1). Among the conserved amino acids, PelC shows an Arg residue (Arg-158) in an identical position to Arg-132 of the mature pectate lyase from Bacillus sp. KSM-P15, which is proposed to initiate proton abstraction during the β-elimination catalytic mechanism (Akita et al., 2001). The three-dimensional structure of PelC was predicted by the Swiss pdb viewer program. The enzyme folds as an eight-turn right-handed parallel β-helix, similarly to family 3 pectate lyases.

Substrate specificity

The cloned pectate lyase was purified by His-tag affinity chromatography, the His-tag was removed and the properties of the enzyme were studied. Analysis by SDS-PAGE, IEF and zymograms showed that PelC has an apparent molecular mass of 23 kDa (Fig. 2) and a pI of 8.6 (data not shown), in good agreement with the predicted values of 24281 Da and 8.55, respectively. The influence of substrate methylation on enzymic activity was tested by evaluating the activity of the purified enzyme on polygalacturonic acid and on pectins of increasing degree of methyl esterification. Release of esterified methyl groups from pectin by saponification can take place after incubation at pH 10 for longer than 10 min (Brown et al., 2001). To avoid this phenomenon, activity was determined in 2.5 min assays in which enzyme samples, substrates and buffer at pH 10 were mixed immediately before the assays. PelC exhibited activity on all substrates tested and showed maximum activity on pectin with a low degree of methylation (22 % esterified) (Fig. 3). On polygalacturonic acid, and pectins of 64 % and 89 % methyl esterification, the enzyme showed 29, 87 and 43 %, respectively, of maximum activity.

To analyse the influence of saponification of pectins on activity evaluation, new assays were performed on substrates that had been kept with buffer at pH 10 for more than 1 h. Under these conditions PelC showed maximum activity on 64 % esterified pectin, while on polygalacturonic acid, 22 % and 89 % esterified pectins, 14, 42 and 97 %, respectively, of maximum activity was found. The results indicated that saponification can give rise to false high activity results on pectins, and for this reason standard activity assay conditions were established in which buffer at pH 10 and the substrates were mixed just before determination of activity.

Pectate and pectin lyases can be distinguished by the absolute Ca2+ requirement for catalysis of pectate lyases versus the lack of Ca2+ requirement by pectin lyases (Jurnak et al., 1996). Analysis of the effect of Ca2+ on PelC activity showed that the enzyme exhibited maximum activity at 2 mM CaCl2, while without Ca2+ addition no activity on polygalacturonic acid and very little activity on pectins were found (Fig. 3). The activity found on pectin without
exogenous Ca\(^{2+}\) could result from the presence of trace amounts of contaminant Ca\(^{2+}\) ions in this substrate. In fact, activity was not detected when the chelator EDTA was included in the assays. The absolute requirement of Ca\(^{2+}\) ions for activity indicates that PelC is a pectate lyase, although, unusually, it shows higher activity on pectins than on polygalacturonic acid and shows activity on highly methylated pectins.

Comparison to PelA from *P. barcinonensis*

The high activity of PelC on pectins resembled that of PelA from *P. barcinonensis*. This enzyme had been previously characterized from crude cell extracts from the recombinant clone *E. coli/pP22* (Soriano et al., 2000). To compare the two pectate lyases more accurately, PelA was purified by His-tag affinity chromatography, and its properties were analyzed.
after removal of the His-tag (Fig. 2). Activity of purified PelA on substrates of different degree of methyl esterification was evaluated. PelA showed maximum activity on polygalacturonic acid, although very similar activity (99% of maximum) was found on 22% esterified pectin (Fig. 3). On highly methylated pectin (89% esterification) the enzyme showed 39% of maximum activity. Activity of the enzyme on 22% esterified pectin (89% esterification) the enzyme showed 39% of maximum activity. Activity of the enzyme on all these substrates showed an absolute requirement of Ca$^{2+}$, as no activity was found in the presence of EDTA. Maximum activity was found when 0.5 mM CaCl$_2$ was added to the assays (Fig. 3).

The effect of metal ions other than Ca$^{2+}$ was analysed by performing activity assays in the presence of metal ions at 1 mM concentration, without Ca$^{2+}$ added. In the case of PelA, activity was undetectable or very low in all assays, indicating that none of the ions studied could replace Ca$^{2+}$. However, when PelC activity was assayed in the presence of 1 mM Hg$^{2+}$, similar activity to control assays was found, suggesting that Hg$^{2+}$ could replace Ca$^{2+}$. When the assays were performed in the simultaneous presence of Ca$^{2+}$ and Hg$^{2+}$, each at 1 mM, a stimulation of PelC activity (120% of control) was found.

The effect of pH and temperature on activities of PelC and PelA was determined. The two enzymes exhibited activity only in a narrow alkaline pH range, with maximum activity at pH 10 (Fig. 2). PelC showed maximum activity at 65°C, while the optimum temperature for PelA activity was 55°C (Fig. 2). Stability assays showed that PelC was stable for at least 24 h at 50°C in buffers at pH 7, whereas PelA showed only 40% of initial activity after incubation under these conditions. When stability assays were performed at 50°C in buffers at pH 10, PelC showed 58% of initial activity after 24 h incubation, while PelA was completely inactivated by this treatment.

The kinetic parameters of purified pectate lyases on pectin and polygalacturonic acid were determined. PelC showed a $V_{max}$ of 256.7 ± 23.9 units mg$^{-1}$ and a $K_m$ of 0.13 ± 0.03 mg ml$^{-1}$ on 22% esterified pectin, while on polygalacturonic acid it showed a $V_{max}$ of 59.6 ± 14.6 units mg$^{-1}$ and a $K_m$ of 0.15 ± 0.06 mg ml$^{-1}$ (means ± SE, n = 3). Kinetic parameters of PelA on 22% esterified pectin were $V_{max}$ 102.4 ± 5.8 units mg$^{-1}$ and $K_m$ 0.14 ± 0.02 mg ml$^{-1}$, while on polygalacturonic acid they were $V_{max}$ 95.4 ± 8.3 units mg$^{-1}$ and $K_m$ 0.16 ± 0.04 mg ml$^{-1}$ (means ± SE, n = 3).

**Analysis of reaction products**

To determine the mode of action of PelC and PelA, the reaction products obtained from various substrates were characterized by TLC. Both enzymes produced a mixture of oligomers from polygalacturonic acid (Fig. 4). The main product released from this substrate by PelC showed a mobility corresponding to unsaturated trigalacturonic acid. Unsaturated digalacturonic acid and oligomers of higher degree of polymerization were also released by the enzyme. PelA produced a mixture of reaction products from polygalacturonic acid similar to that produced by PelC, although the main products were oligomers longer than unsaturated trigalacturonic acid. Analysis of the reaction products from pectin showed that PelC and PelA released a mixture of oligomers with a similar pattern to that obtained from polygalacturonic acid. In contrast to the similar action of PelC and PelA on polygalacturonic acid and pectin, the enzymes behaved differently on trigalacturionate. PelC degraded trigalacturionate to galacturonic acid and unsaturated oligomers with a similar pattern to that obtained from polygalacturonic acid.
digalacturonic acid, whereas PelA was not active on this substrate.

**DISCUSSION**

We have identified and characterized a new pectin-depolymerizing enzyme from *B. subtilis* that cleaves bonds by β-elimination. The enzyme does not show homology to the two pectinases, a pectate lyase (Nasser et al., 1993) and a pectin lyase (Sakamoto et al., 1996), characterized up to now from this species. As no hydrolases acting on pectic polymers have been described in *B. subtilis*, the enzyme constitutes the third pectin depolymerase characterized in this species, and has been denominated PelC. The fact that until our work only two pectinases had been identified from *B. subtilis* contrasts with the pectin-depolymerizing activity of this species (Chesson & Codner, 1978) and the multiplicity of pectinases described in pectin-degrading microorganisms, which show complex sets of enzymes. In this way, at least eight pectate lyases have been cloned and characterized in *Erwinia chrysanthemi* (Shevchik et al., 1999b), while *Aspergillus niger* contains several pectin lyases, a pectate lyase and a family of polygalacturonases (Pařeníková et al., 2000). We believe that *B. subtilis*, besides the three pectin-degrading enzymes characterized up to now, has several as yet unidentified pectinases and may exhibit a complex set of enzymes for pectin degradation.

One of the most notable features of PelC is its activity on both polygalacturonic acid and highly methylated pectin. Although many pectate lyases show maximum activity on polygalacturonic acid, some are most active on low methylated substrates (7–22 % esterification) (Tardy et al., 1997). The activity of these enzymes on pectin could indicate that they are pectin lyases rather than pectate lyases, although a clear difference between the two types of enzymes is the absolute Ca²⁺ requirement for catalysis of pectate lyases, while pectin lyases do not require Ca²⁺ (Jurnák et al., 1996). Our results show that *B. subtilis* PelC requires added Ca²⁺ for activity on pectic substrates, indicating that it is a pectate lyase. The enzyme shows maximum activity on 22 % methylated pectin, similar to what has been reported for *Er. chrysanthemi* PelB (Tardy et al., 1997). However, in contrast to known pectate lyases, PelC shows high activity (43 % of maximum activity) on 89 % methylated pectins. This is a unique trait among pectate lyases characterized to date, which are not active or show very low activity on highly methylated pectins (Benen & Visser, 2003; Hugouvieux-Cotte-Pattat et al., 1996), although some pectate lyases of family 3, such as Pel from *Er. chrysanthemi* and PelB from *Er. carotovora*, exhibit maximum activity on 45 % and 68 % esterified pectin, respectively (Shevchik et al., 1998; Heikinheimo et al., 1995). Pectate lyase PelA from *P. barcinonensis* (Soriano et al., 2000), the enzyme that led to identification of PelC, resembles this enzyme in its high activity on highly esterified pectin (39 % of maximum activity on 89 % methylated pectin). This is a difference between these two enzymes (PelC and PelA) and the pectate lyases described up to now.

Sequence comparison with proteins contained in databases revealed that PelC from *B. subtilis* and PelA from *P. barcinonensis* belong to family 3 pectate lyases. Sequence alignment of enzymes of this family has identified four blocks of conserved residues, proposed as signatures of pectate lyases of family 3 (Shevchik et al., 1997). These blocks of residues are also found in pectate lyases from plant-parasitic nematodes (Huang et al., 2005). However, the two pectate lyases characterized in this work, together with pectate lyase from *Bacillus* sp. P-2850 (accession no. Q8LOR5) and pectate lyase from *Bacillus* sp. KSM-P15 (Hatada et al., 2000) do not show the fourth block of conserved residues. In the corresponding area of these four enzymes (residues 172–221 in the case of PelC) analysis with ProDom at NCBI (Servant et al., 2002) has identified domain ID:PD346936, not found in other enzymes. The four pectate lyases show high homology (more than 50 % identity in amino acid sequence), similar size (221–224 amino acids), and low content of cysteine residues.
residues (1–3) compared to the rest of the enzymes of family 3, which show a higher cysteine content [12 residues in the case of PelB from Fusarium solani (Guo et al., 1995)]. The results show that PelC from B. subtilis and PelA from P. barcinonensis, together with pectate lyase from Bacillus sp. P-2850 and pectate lyase from Bacillus KSM-P15, constitute a subgroup of homology among family 3 pectate lyases. A dendrogram of enzymes of family 3 generated with CLUSTAL W (Thompson et al., 1994) showed that these four pectate lyases cluster separately from the rest of enzymes of family 3 (data not shown), and form a subgroup of pectate lyases from saprophytic bacteria in a family where most of the enzymes belong to pathogenic micro-organisms. Analysis of the hypothetical protein deduced from the yvpA gene from B. licheniformis (accession no. Q65EF5) reveals it shares all the common traits of the enzymes of the subgroup identified and clusters with them.

As mentioned above, PelC and PelA have as a distinctive character their high activity on pectin with a high degree of methyl esterification. This property is not shared by the other enzymes of the homology subgroup. While activity results of pectate lyase from Bacillus sp. P-2850 have not been reported, pectate lyase from Bacillus sp. KSM-P15 shows a clear preference for polygalacturonic acid and very low activity on methylated pectins (Kobayashi et al., 1999). Despite their similarity, PelC and PelA show some differences regarding substrate specificity. PelC shows a clear preference for 22 % methylated pectin, whereas PelA shows similar activity on polygalacturonic acid and 22 % esterified pectin. Study of the mode of action on polymeric substrates showed that PelC and PelA have an endo mode of action, producing a mixture of degradation products. A similar mode of action has been reported for pectate lyases of family 1, such as pectate lyase A from Clostridium cellulovorans (Tamaru & Doi, 2001), family 3, such as Pell from Er. chrysanthemi (Shevchik et al., 1997), and family 9, such as Pell from Er. chrysanthemi (Lojkowska et al., 1995). In contrast, PelX and PelW from Er. chrysanthemi (Shevchik et al., 1999a, b), which belong to families 9 and 2, respectively, show an exo mode of action, releasing unsaturated digalacturonate as the only degradation product from polygalacturonic acid. PelA and PelC differed in their activity on trigalacturonate: PelA did not degrade this substrate, whereas PelC cleaved it to galacturonic acid and unsaturated digalacturonate. The lack of trigalacturonate degradation by PelA is similar to that reported for endopectate lyase A from Clostridium cellulovorans (Tamaru & Doi, 2001) and to the requirement for substrates longer than a trimer for effective catalysis of several endopectate lyases from Er. chrysanthemi (Roy et al., 1999). However, degradation of trigalacturonate by PelC resembles the results described for exopectate lyases PelX and PelW of Er. chrysanthemi (Shevchik et al., 1999a, b). The scarcity of reports about the use of trigalacturonate as a substrate by pectate lyases makes it difficult to understand the significance of the different behaviour of PelA and PelC on this oligomer.

In summary, we have purified and characterized two novel pectate lyases, PelC from B. subtilis and PelA from P. barcinonensis, which differ from known pectate lyases in their high activity on highly methylated pectin. The characterized enzymes, together with pectate lyase from Bacillus sp. P-2850 and pectate lyase from Bacillus sp. KSM-P15, constitute a subgroup of highly homologous enzymes in family 3. The biochemical properties of the four pectate lyases need to be studied in more detail to identify common features of this subgroup of enzymes, which to our knowledge are the

![Fig. 4. Reaction products from polygalacturonic acid, pectin and trigalacturonate. Samples of 1·2 μg purified PelC or 2·6 μg purified PelA were incubated in 500 μl assay mixture containing 0·15 % substrate at 55 °C (PelC) or 40 °C (PelA) and samples were taken for analysis of the reaction products at time 0 (lanes 0), 30 min (lanes 1), 3 h (lanes 2) and 24 h (lanes 3). PGA, polygalacturonic acid; pectin, 22 % methylated citrus pectin (PelC) or 89 % methylated citrus pectin (PelA); G₃, trigalacturonate. Lanes M contain size markers: galacturonic acid (G), digalacturonate (G₂) and trigalacturonate (G₃) (Sigma).](http://mic.sgmjournals.org)
only pectate lyases from non-pathogenic micro-organisms classified in family PL3.

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