

## Characterization of Two *Paenibacillus amylolyticus* Strain 27C64 Pectate Lyases with Activity on Highly Methylated Pectin<sup>∇†</sup>

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**Two pectate lyases were identified from *Paenibacillus amylolyticus* 27C64; both enzymes demonstrated activity on methylated pectin in addition to polygalacturonic acid. *PelA* is in a subclass of the pectate lyase family III. *PelB* shows some features of pectate lyase family I but is highly divergent.**

Pectinases have many industrial applications, including uses in food and textile production (9, 12). Additionally, pectinases are important for the degradation of biomass, where pectin can comprise a significant portion of plant structure (5, 6). The degradation of pectin requires methylesterases and depolymerases. Pectin methylesterases are responsible for the hydrolysis of methylester linkages from the polygalacturonic acid (PGA) backbone (24), while pectin depolymerases act upon the polygalacturonate backbone and belong to one of two families, polygalacturonases or lyases. Polygalacturonases hydrolytically cleave the polygalacturonate chain, while lyases

cleave by  $\beta$ -elimination, giving a  $\Delta 4,5$ -unsaturated product (10, 19). There are two types of lyases: pectate lyases (PLs), which cleave unesterified polygalacturonate, and pectin lyases, which cleave methylesterified pectin.

*Paenibacillus amylolyticus* strain 27C64, isolated from the larval hindgut of the aquatic crane fly, *Tipula abdominalis*, possesses a wide range of lignocellulose-degrading enzymes. This study describes two pectate lyases from *P. amylolyticus* that display unusual activity by combining traits of pectate and pectin lyases (2, 7, 21, 22).

**Identification of *pelA* and *pelB*.** A library containing 2- to

TABLE 1. Cloning strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics	Reference or source
Bacterial strain <i>Escherichia coli</i> DH5 $\alpha$	F <sup>-</sup> <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> $\phi 80dlacZ\Delta M15$ $\Delta(lacZYA-argF)U169 hsdR17(r_K^- m_K^+) \lambda^-$	16
Plasmids		
pUC19	<i>lacZ</i> $\alpha^+$ ; Ap <sup>r</sup>	Invitrogen (Carlsbad, CA)
pEDH13C2	pUC19 derivative; <i>P. amylolyticus</i> DNA fragment with <i>pelA</i>	This study
pEDH27	pUC19 derivative; <i>pelA</i> <sup>+</sup>	This study
pUC19-19F6	pUC19 derivative; <i>P. amylolyticus</i> DNA fragment with <i>pelB</i>	This study
pWEB1	pUC19 derivative; <i>pelB</i> <sup>+</sup>	This study
Oligonucleotides		
PLAscF (for <i>pelA</i> )	5'-GTACAGGGCCCGGATCCTTGACATGATAGAAGCACTCTAC TATATTCTAGTGCTTCTACGGTTCTGTGGGACAA-3'	This study
PLAscR (for <i>pelA</i> )	5'-CGATCAAGCTTGGGCCCGAGCGCCGCTCGAGTCCACAT GGTTTGGAGCATTTTCG-3'	This study
ScpelBF (for <i>pelB</i> )	5'-GCAGTGAGCTCTTGACATGATAGAAGCACTCTACTATATT CTAGTTATACTTATCGGGAGGAATCG-3'	This study
ScpelBR (for <i>pelB</i> )	5'-CATGGGATCCCGGAGCGCTTAACCTTAGTAACTC-3'	This study

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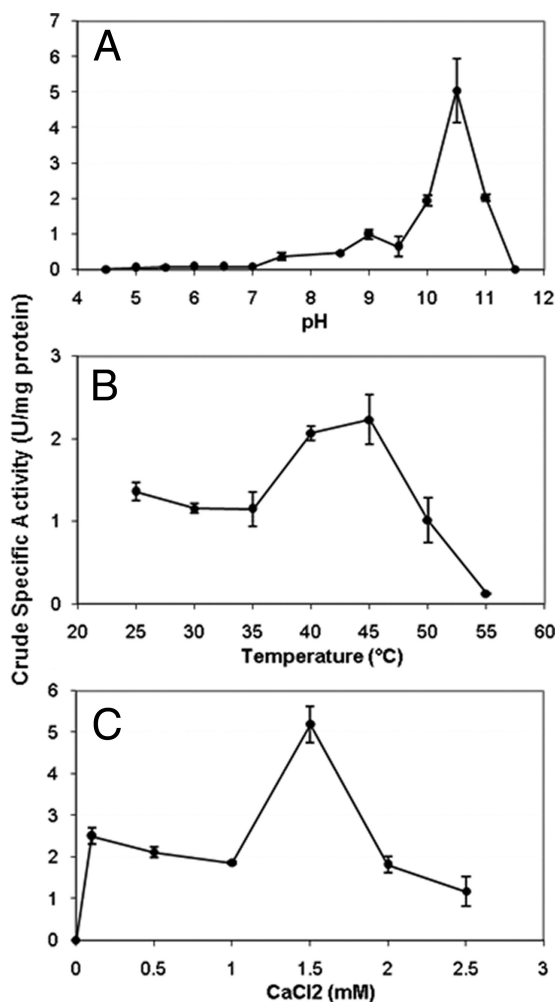


FIG. 1. *P. amylolyticus* strain 27C64 PelA optima for pH (A), temperature (B), and CaCl<sub>2</sub> (C). The pH optimum was determined at 40°C with 1 mM CaCl<sub>2</sub>, the temperature optimum was determined at pH 10.5 in a range of 25 to 55°C, and the CaCl<sub>2</sub> concentration optimum was determined at pH 10.5 and 45°C in a range of 0 to 2.5 mM. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol Δ4,5-unsaturated product from PGA per minute under assay conditions; specific activity is reported as U/mg protein.

5-kb chromosomal fragments of *P. amylolyticus* strain 27C64 was constructed in *Escherichia coli* DH5α. Two pectinase-positive clones were identified after screening approximately 6,000 clones on polygalacturonase medium (23) and subcloned for characterization (Table 1). The first clone carried a 2-kb insert with an open reading frame (ORF) of 669 bp (*pelA*, GenBank accession no. GU289919); a putative ribosomal binding site and promoter was located upstream of the ATG start codon. The deduced protein sequence of the ORF is 222 amino acids and contains an N-terminal region with features of a *Bacillus* signal peptide, the most likely cleavage site being between amino acids 26 and 27 (17).

The second pectinase-positive clone had a 1.5-kb insert with a single ORF of 1,176 bp (*pelB*, GenBank accession no. GU289920). Located upstream of *pelB* was a putative ribosomal binding site and promoter element. The deduced protein sequence of the ORF is 302 amino acids and contains an

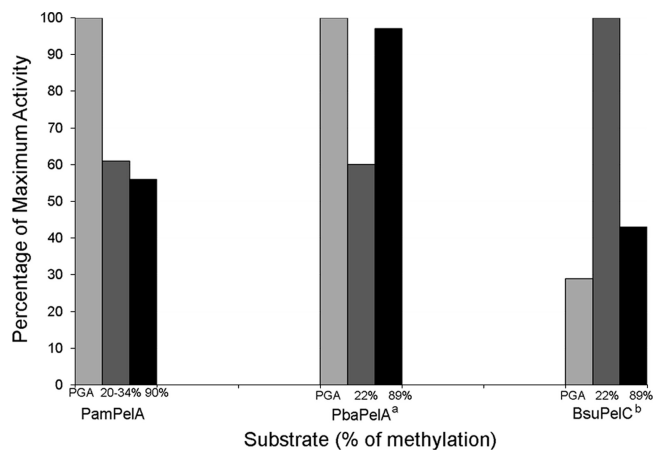


FIG. 2. Activity on different pectic substrates for *P. amylolyticus* strain 27C64 pectate lyase A (PamPelA), *P. barcinonensis* pectate lyase A (PbaPelA), and *B. subtilis* pectate lyase C (BsuPelC). a, data from reference 21; b, data from reference 22. *P. amylolyticus* strain 27C64 PelA, *P. barcinonensis* PelA, and *B. subtilis* PelC substrate utilization ranges, with activity on PGA as well as pectin with any degree of methylation, are unique among the pectate lyases described to date.

N-terminal region with features of a *Bacillus* signal peptide, the most likely cleavage site being between amino acids 30 and 31 (17).

**Identification and characterization of PelA.** By protein-protein BLAST (blastp) with the NCBI database (1), PelA exhibited homology to pectate lyases within family III (PL3). PelA was 95% identical to PelA from *Paenibacillus barcinonensis* (21), 78% identical to *Bacillus* sp. KSM-P15 pectate lyase (7), 55% identical to *Bacillus subtilis* PelC (22), 54% identical to *Bacillus licheniformis* YypA, and 53% identical to *Bacillus* sp. P-2850 pectate lyase (see Table S1 in the supplemental material for GenBank accession numbers). Three of four signature blocks of conserved residues for PL3 enzymes (20) are found in PelA, but as in its homologous enzymes, the fourth block of residues is not conserved; it is replaced by another domain not found in other pectate lyases (22) (see Fig. S1). PelA appears to belong to a subgroup of family PL3 enzymes from saprophytic bacteria (22) which includes *P. barcinonensis* PelA, *Bacillus* sp. KSM-P15 PL, *B. subtilis* PelC, *B. licheniformis* YypA, and *Bacillus* sp. P-2850 PL.

PelA exhibited pectate lyase activity on polygalacturonic acid (PGA) but did not show xylanase or cellulase activity with model substrates and liberation of reducing sugars. Pectate lyase assays were performed as described previously (3, 21) with *E. coli* DH5α clone cell extracts prepared by sonication, and pH, temperature, and CaCl<sub>2</sub> optima were determined (Fig. 1). *E. coli* DH5α(pUC19) extracts had no detectable pectate lyase activity, and PelA was closely related to characterized proteins; therefore, further studies with purified enzyme were not performed. CaCl<sub>2</sub> was necessary for activity, as it is for all known pectate lyases (11).

Activity of PelA on citrus pectin was also investigated (Fig. 2), and assays with 20 to 34% and 90% methylated citrus pectin demonstrated activity at 61% and 56% of the maximum activity on PGA, respectively (Fig. 2). The high activity of PelA on both PGA and pectins with low and high levels of methyl-

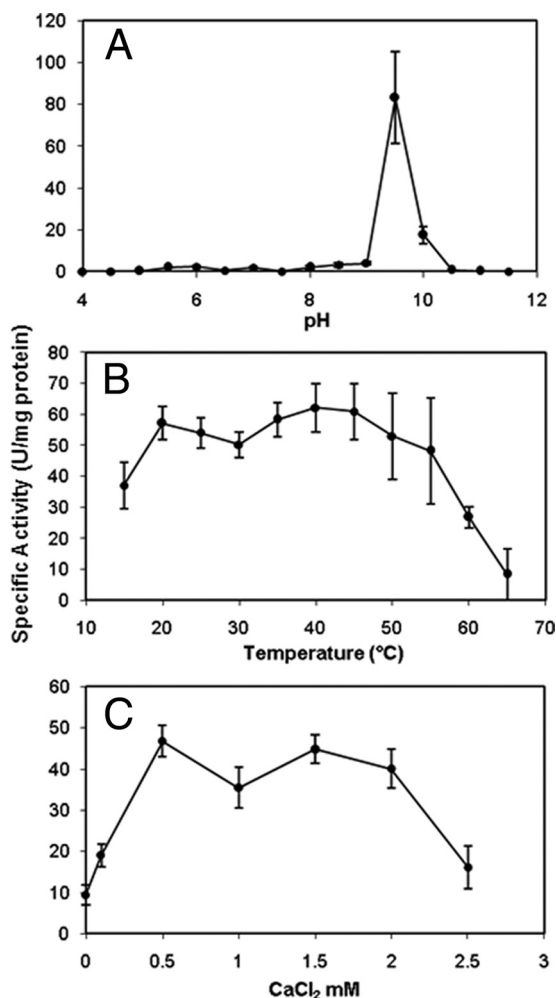


FIG. 3. *P. amylolyticus* strain 27C64 PelB optima for pH (A), temperature (B), and CaCl<sub>2</sub> (C). The pH optimum was determined at 40°C with 1 mM CaCl<sub>2</sub>, the temperature optimum was determined at pH 9.5 in a range of 15 to 65°C, and the CaCl<sub>2</sub> concentration optimum was determined at pH 9.5 and 55°C in a range of 0 to 2.5 mM.

ation, although also observed for PelA from *P. barcinonensis* and PelC from *B. subtilis* (21, 22), is rare among the pectate lyases described to date.

**Identification and characterization of PelB.** PelB showed low homology to family I pectate lyases (PL1) with 28% identity to *Bacillus* sp. YA-14 PelK (13), *B. licheniformis* Pel (18), and *B. subtilis* reference strain 168 Pel (15); 27% identity to *Thermotoga maritima* PelA (14); and 26% identity to *B. subtilis* BS-2 Pel and *Bacillus amyloliquefaciens* TB-2 Pel using protein-protein BLAST (blastp) with the NCBI database (1) (see Table S1 in the supplemental material for GenBank accession numbers). Compared with other pectate lyases, PelB contains all three of the conserved calcium binding sites (25), six of the seven conserved thermostable PL1 sites (Gly47, Val81, Ile83, Leu87, Arg157, and Val214), all of the three conserved catalytic sites, and 9 of 10 invariant residues found in all pectate lyases (see Fig. S2).

Three conserved sequence patterns are also typically contained in pectate lyases: vWiDH, VxxRxPxxRxGxxHxxxxN,

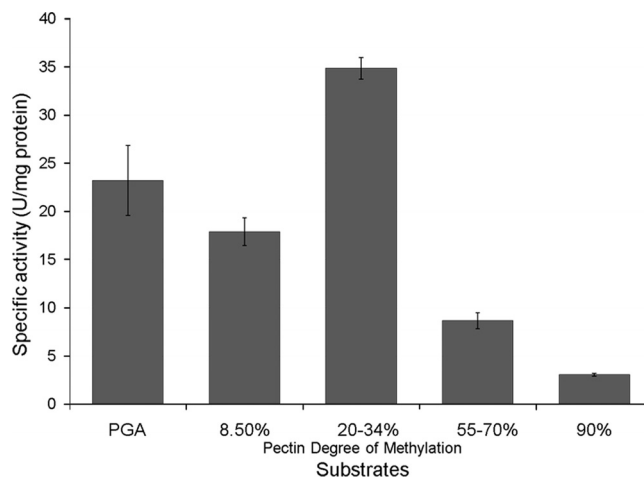


FIG. 4. *P. amylolyticus* strain 27C64 pectate lyase B activity on different pectic substrates. PelB showed the highest activity on 20 to 34% methylated pectin but retained 67%, 51%, 25%, and 1% of its maximum activity on polygalacturonic acid and 8.5%, 55 to 70%, and 90% methylated pectin, respectively, providing evidence that PelB is active on PGA as well as highly methylated pectin.

and AxDIKGxxxxVTxS. PelB and its closest homologs contain the vWiDH and VxxRxPxxRxGxxHxxxxN regions, while the final conserved sequence, AxDIKGxxxxVTxS, is mostly conserved in PelB.

Four highly conserved consecutive Asn ladder positions which help to stabilize the  $\beta$  bend of the protein structure are found in all PL enzymes. PelB contains only half of the conserved Asn ladders, whereas all of the other enzymes being compared to PelB in this study contain all four of the conserved Asn ladders (8).

Pectate lyase assays for PelB were performed as with PelA but with *E. coli* DH5 $\alpha$  clone concentrated supernatant. Denaturing SDS-PAGE separation and native zymogram analysis (4) of *E. coli* DH5 $\alpha$ (pWEB1) supernatant with ImageJ analysis showed that PelB was 86% of the supernatant protein, and it was the only band with pectinase activity on zymograms. PelB pH, temperature, and CaCl<sub>2</sub> optima were determined (Fig. 3). The enzyme preparation contained 0.115 mM calcium, but dependence on calcium was determined by studies with 0.5 M EDTA, in which no activity was detected (data not shown).

PelB was evaluated for its ability to cleave PGA and methylated pectin by using a range of pectic substrates (Fig. 4). PelB showed the highest activity on 20 to 34% methylated pectin but retained 67%, 51%, 25%, and 1% of its maximum activity on polygalacturonic acid and 8.5%, 55 to 70%, and 90% methylated pectin, respectively.

*P. amylolyticus* 27C64 PelA and PelB are the first pectate lyases described in *P. amylolyticus* and show an unusual combination of pectate lyase and pectin lyase activity by degrading both polygalacturonic acid and highly methylated pectin, respectively. A subgroup of PL family III, which now includes PelA, demonstrates this broad substrate specificity; however, enzymes of closest homology or shared structure to PelB do not. While PelB has structural features in line with pectate lyase family I, it is missing some of the conserved amino acid regions and one of the three extracellular pectate lyase super-

family conserved amino acid regions (8, 25). Recently sequenced genomes and metagenomes suggest that enzymes with significant homology to PelB exist. The characterization of these enzymes will determine if they share the substrate specificity of PelB and if they form a new subclass or family of pectate lyases.

**Nucleotide sequence accession numbers.** Sequences for *pelA* and *pelB* are available in GenBank under accession numbers GU289919 and GU289920, respectively.

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