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Highly alkaline pectate lyase Pel-4A from alkaliphilic *Bacillus* sp. strain P-4-N: its catalytic properties and deduced amino acid sequence

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Abstract The gene for a highly alkaline pectate lyase, Pel-4A, from alkaliphilic *Bacillus* sp. strain P-4-N was cloned, sequenced, and overexpressed in *Bacillus subtilis* cells. The deduced amino acid sequence of the mature enzyme (318 amino acids, 34805 Da) showed moderate homology to those of known pectate lyases in the polysaccharide lyase family 1. The purified recombinant enzyme had an isoelectric point of pH 9.7 and a molecular mass of 34 kDa, and exhibited a very high specific activity compared with known pectate lyases reported so far. The enzyme activity was stimulated 1.6 fold by addition of NaCl at an optimum of 100 mM. When Pel-4A was stored at 50°C for 60 h, striking stabilization by 100 mM NaCl was observed in a pH range from 5 to 11.5, whereas it was stable only around pH 11 in the absence of NaCl.

Key words Alkaliphile · Pectate lyase · Cloning · *Bacillus* · Salt dependency

Introduction

Pectinolyic enzymes have been studied with special focus on soft-rot diseases of higher plants (Rombouts and Pilnik 1980), on processing aids for the food and beverage industries (Alkorta et al. 1998), and also on retting processes of plant fibers (Henriksson et al. 1999). Pectate lyase (Pel; pectate transeliminase, EC 4.2.2.2) is the pectin depolymerizing enzyme that cleaves α-1,4-galacturonosidic linkages of polygalacturonic acid (PGA) by a *trans*-elimination mechanism and requires Ca^{2+} ions for activity (Rombouts and Pilnik 1980). Many genes for microbial Pels have been cloned and sequenced, and the enzymes form a superfamily based on their deduced amino acid (aa) sequences (Henrissat et al. 1995; Heffron et al. 1995). The tertiary structures of PelC (Yoder et al. 1993) and PelE (Lietzke et al. 1994) from *Erwinia chrysanthemi* EC16 and BsPel from *Bacillus subtilis* SO113 (Pickersgill et al. 1994) have been solved, and they have a structural topology of parallel β-strands with a large right-handed coil (Heffron et al. 1998).

To cope with extremes of alkaline pH or high Na concentration, alkaliphilic *Bacillus* spp. exoproduce various alkaline hydrolases, including Pels, some of which are of industrial importance (Horikoshi 1999). The surface loops of Pel are known to alter the surface charge distribution, overall shape, and catalytic properties (Lietzke et al. 1994). Shortening and aa substitutions of loops and core structure of Pel might occur during the process of adaptation to an alkaline environment. We are now collecting the data of catalytic properties and aa sequences of *Bacillus* Pels to construct a molecular phylogenetic tree of highly alkaline Pels and related less alkaline enzymes. We have already characterized highly alkaline Pels, including Pel-7 (Kobayashi et al. 1999) in the polysaccharide lyase family 1 from alkaliphilic *Bacillus* sp. strain KSM-P7 and three Pel isoenzymes, Pel-15 (family 3) (Hatada et al. 2000), Pel-15E (family 10) (Sawada et al. 2000), and Pel-15H (family 9) (Ogawa et al. 2000), from alkaliphilic *Bacillus* sp. strain KSM-P15. The deduced aa sequences and catalytic properties of the highly alkaline Pels were different from those of less alkaline enzymes from neutrophilic bacteria and fungi reported so far. During the screening of alkaline pectinases for fiber retting, we also isolated two different genes for Pels, designated *pel*-4A and *pel*-4B, from alkaliphilic *Bacillus* sp. strain P-4-N, which produces an alkaline pectinase (Horikoshi 1972). Here we describe the activation and stabilization by NaCl of the *pel*-4A gene product Pel-4A and compare the deduced aa sequence of the enzyme with those of other enzymes belonging to the large Pel superfamily.

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Materials and methods

Bacterial strains and propagation

The source of the gene examined in this study was alkaliphilic *Bacillus* sp. strain P-4-N. The organism was propagated, with shaking, at 37°C for 15 h in an alkaline liquid medium (pH 9.5) composed of (w/v) 3.0% pectin (Sigma), 0.5% polypepton S (Nippon Pharmaceutical, Tokyo, Japan), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 5H_2O$, and 1.0% Na_2CO_3 (separately autoclaved).

Isolation of DNA, transformation, and sequencing

Preparation of plasmid and genomic DNAs, restriction digestion, ligation, and transformation of *Escherichia coli* HB101 and *B. subtilis* ISW1214 cells were done as described (Hatada et al. 2000). Transformed *E. coli* cells were grown on Luria-Bertani agar containing 0.5% (w/v) PGA (Sigma) and 50 μg ml^{-1} ampicillin. Pel-positive clones formed clear halos around the colonies. Sequencing of the plasmid DNA extracted from the clones was done using a DNA sequencing kit-dye terminator cycle sequencing ready reaction (Perkin-Elmer, Norwalk, CT, USA) and an automated DNA sequencer (model 377; Perkin-Elmer).

Cloning of the gene for Pel-4A

The *pel*-4A gene was first cloned by the conventional shotgun method using *Bam*HI and *Hin*dIII into the *Bam*HI/*Hin*dIII site of pUC18. A constructed plasmid containing a 5.5-kb DNA fragment in a Pel-positive clone was truncated, after linearization with *Hin*dIII, by partial digestion with a *Bal*31 nuclease (Takara). The digests were blunt-ended by T4 DNA polymerase (Takara) and then digested with *Bam*HI. The truncated DNA fragments of various lengths were subcloned into the *Bam*HI/*Sma*I site of pUC18. After transformation of *E. coli* HB101with the ligation mixtures, one Pel-positive clone was obtained. The size of the cloned DNA fragment was approximately 2.3 kb when measured on a 1% agarose gel electrophoresis.

Expression of the gene for Pel-4A in *B. subtilis* cells

The *pel*-4A gene was amplified by PCR with a sense primer and an antisense primer, 5′-TTGTAAACGAC GGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′, which were designed from the upstream and downstream regions of the multicloning site of pUC18. The primers were prepared on a DNA synthesizer (model 392A; Applied Biosystems, Foster City, CA, USA). PCR was done in a DNA thermal cycler (model 480; Perkin-Elmer) by using the two primers plus pUC18 containing the 2.3-kb DNA fragment as template (5 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C). The amplified DNA fragment of approximately 2.3 kb was then cloned into the *Sma*I site of pHY300PLK (Ishiwa and Shibahara-Sone 1986) and *B. subtilis* ISW1214 cells were transformed with the constructed plasmid. The transformed cells were grown, with shaking, at 30°C for 2 days in a liquid medium composed of (w/v) 4% maltose, 2.8% polypepton S, 0.5% fish meal extract (Wako, Osaka, Japan), 0.05% yeast extract (Difco), 0.1% KH_2PO_4, 0.001% $MgSO_4 \cdot 7H_2O$, and 7.5 μg ml^{-1} tetracycline (Sigma).

Purification of the recombinant enzyme

All purification steps were performed below 5°C. The culture broth (40 ml) was centrifuged at 10,000 *g* for 15 min to remove cells and debris, and an equal volume of distilled water was added to the supernatant to lower its ionic strength. The diluted solution (0.48 mS cm^{-1}) was directly applied on a column (2.5 × 10 cm) of Super Q Toyopearl 650M (Tosoh) that had been equilibrated with 50 mM Tris-HCl buffer plus 5.0 mM $CaCl_2$ (pH 8.0; buffer A). Pel activity was passed through the column by elution with buffer A. The nonadsorbed fractions were pooled (80 ml) and diluted to 100 ml with distilled water. After the solution was applied to a column (2.5 × 11 cm) of SP Toyopearl 650M (Tosoh) equilibrated with buffer A, the column was initially washed with 100 ml of buffer A, and proteins were then eluted with a 500-ml linear gradient of 0 to 250 mM KCl in the same buffer. Pel activity was eluted between 50 and 60 mM KCl. The active fractions were combined (32 ml) and concentrated by ultrafiltration with a YM-3 membrane (Amicon, Beverly, MA, USA) to 1.25 ml. The concentrate was then applied to a column (1.5 × 73 cm) of Bio-Gel-A0.5m (Bio-Rad, Hercules, CA, USA), and the proteins were eluted with 10 mM Tris-HCl buffer (pH 8.0) plus 100 mM KCl and 5 mM $CaCl_2$. The eluted fractions containing Pel activity were combined and concentrated with a YM-membrane to 3.5 ml. The concentrated extract was applied to a column (1.5 × 12.5 cm) of hydroxylapatite (Bio-Rad) equilibrated with 50 mM phosphate buffer (pH 7.0), and the column was washed first with 70 ml of the same buffer. Proteins were eluted with a 200-ml linear gradient of 50 to 200 mM phosphate buffer. The active fractions eluted between 180 and 190 mM phosphate buffer were pooled and concentrated with a YM membrane and stored in 20% (v/v) glycerol until use. When the enzyme was purified in 10 mM, instead of 50 mM, Tris-HCl buffer (pH 7.5) plus $CaCl_2$, the Pel activity was lost completely.

Enzyme assays and electrophoresis

Pel activity was routinely measured at 30°C and at pH 10.5 in 50 mM glycine-NaOH buffer containing 0.6 mM $CaCl_2$ and 100 mM NaCl. One unit of enzymatic activity was defined as the amount of protein that produced 1 μmol of unsaturated oligogalacturonides equivalent to 1 μmol of unsaturated digalacturonide, using the molecular extinction coefficient value of 4600 M^{-1} cm^{-1} at 235 nm for the dimer. Protein was determined with a DC-protein assay kit (Bio-Rad) with bovine serum albumin as the standard protein.

Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE were done as described previously (Hatada et al. 2000). SDS-PAGE molecular weight standards (Pharmacia) were used as the marker proteins, which included phosphorylase *b* (97.4 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.1 kDa), and α-lactalbumin (14.4 kDa). Isoelectric focusing (IEF) of proteins was done with a mini IEF Cell (model III; Bio-Rad) with 5% (w/v) acrylamide gel and 3% (v/v) ampholine (pH 8–10.5; Pharmarite; Pharmacia). Cytochrome *c* and its acetylated derivatives (Oriental Yeast) were used as isoelectric point (pI) markers.

Sequencing of the N-terminal region

To analyze the N-terminal aa sequence of mature Pel-4A, the enzyme sample was blotted onto a polyvinylidene difluoride membrane (Pro-sorb; Perkin-Elmer) wetted with methanol. The N-terminal sequence of the protein was determined directly by a pulsed liquid-phase protein sequencer (model 476A; Applied Biosystems).

Nucleotide sequence submission

The nucleotide (nt) sequence data published here have been deposited in the DDBJ, EMBL, and GenBank data banks under accession number AB041769.

Results and discussion

Purification and some properties of recombinant Pel-4A

Because *Bacillus* sp. stain P-4-N exoproduced Pel-4A very poorly, the *pel*-4A gene was successfully cloned and then overexpressed in *B. subtilis* at a level corresponding to about 0.22 g l^{-1}. The recombinant Pel-4A was purified 59 fold to an overall yield of 14%, with a specific activity toward PGA of 943 units (mg protein)$^{-1}$ (Table 1). The purified enzyme was homogeneous as judged by both PAGE and SDS-PAGE. The molecular mass of Pel-4A was approximately 34 kDa by SDS-PAGE. The pI value was about pH 9.7 as estimated by IEF PAGE. The N-terminal aa sequence of the purified Pel-4A was Asn-Ser-Tyr-Ser-Phe-Lys-Ser-Thr-Thr-Gly-Trp-Ala. When NaCl was included in the reaction mixtures at an optimum of 100 mM, the Pel activity was enhanced by 150%–160% relative to the control (without NaCl, taken as 100%). Sodium sulfate at 50 mM also enhanced activity up to the same level (160%). LiCl (100 mM), KCl (100 mM), and K_2SO_4 (50 mM) restored the activity up to 129, 120, and 109, respectively, whereas the activity was inhibited 79% by NH_4Cl (100 mM) and 52% by $(NH_4)_2SO_4$ (50 mM). Pel-4A (0.38 μg ml^{-1}) was incubated at 30°C for 30 min with 100 mM EDTA in 25 mM Tris-HCI buffer (pH 8.0) plus 100 mM NaCl. The activity of the EDTA-treated enzyme was abolished when measured without $CaCl_2$ under the standard conditions of the assay. It was completely restored by addition of $CaCl_2$ at 0.4–0.6 mM. Mn^{2+}, Pd^{2+}, Co^{2+}, Sr^{2+}, and Zn^{2+} ions (at 0.4 mM each) also restored the activity to 20%, 18%, 14%, 13%, and 4% of the original activity, respectively. Mg^{2+}, Ni^{2+}, Fe^{2+}, and Fe^{3+} ions could not substitute for Ca^{2+} ions.

Substrate specificity

When PGA degradation rate at pH 10.5 in the presence of both 0.6 mM $CaCl_2$ and 100 mM NaCl was taken as 100%, the relative rate toward citrus pectins with degrees of esterification of 28%, 31%, 63%, and 93% (Sigma) were 81%, 79%, 37%, and 0%, respectively.

Effects of temperature and pH

The effect of temperature on the Pel-4A activity was examined at pH 10.5 in 50 mM glycine-NaOH buffer with or without NaCl. The apparent optimal temperatures for activity in the absence and presence of NaCl (100 mM) were observed to be around 60°C for both. Arrhenius plots of the reactions showed that the activation energies without NaCl were 64.0 below and –246 kJ mol^{-1} above the break at 64.8°C, whereas those with 100 mM NaCl were 66.1 below and –167 kJ mol^{-1} above the break at 58.1°C (Fig. 1A). The difference in the activation energy beyond the break suggests the thermal stabilization by NaCl. To assess the positive effect of NaCl, Pel-4A was incubated at various temperatures for 15 min in 50 mM glycine-NaOH buffer (pH 10.5) plus 100 mM NaCl and/or 2 mM $CaCl_2$. In the absence of the additives, the activity was gradually reduced by heating above 30°C. In contrast, the enzyme was stable up to 55°, 55°, or 60°C in the presence of either NaCl, $CaCl_2$, or both, respectively (Fig. 1B).

The effect of pH on the Pel-4A activity toward PGA was examined at 30°C in the absence or presence of NaCl. The optimal pH of the nonactivated enzyme was pH 11.5; the NaCl-activated enzyme had an optimal pH of 11 (Fig. 2A). When the enzyme was stored at 5°C for 60 h, striking stabi-

Table 1. Purification of the recombinant pectate lyase expressed in *Bacillus subtilis*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)	Fold
Culture broth	572	9190	16.1	100	1.0
Super Q Toyopearl	106	4990	47.1	55	2.9
SP Toyopearl	5.2	4410	848	48	53
Bio-Gel-A	3.0	2640	880	29	55
Hydroxylapatite	1.4	1320	943	14	59

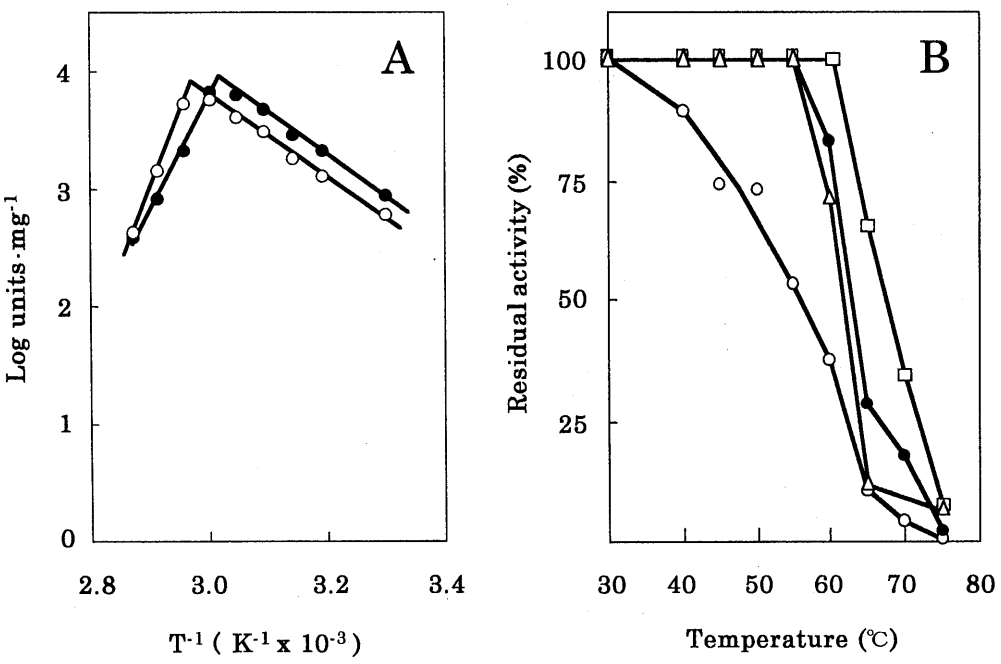

Fig. 1A,B. Effects of temperature and NaCl on activity. **A** Arrhenius plot of the Pel-4A reaction. Assay of Pel activity was done at various temperatures in 50 mM glycine-NaOH buffer (pH 10.5) plus 0.6 mM $CaCl_2$ with 0.02 μg Pel-4A either without (*open circles*) or with (*solid circles*) 100 mM NaCl. **B** Effect on thermal stability. Pel-4A (0.4 μg) was incubated at the indicated temperatures for 15 min in 50 mM Tris-HCl buffer (pH 7.0) in the absence (*open circles*) or presence (*solid circles*) of 100 mM NaCl, 2.0 mM $CaCl_2$ (*open triangles*), or both additives (*open squares*). Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity without heating was taken as 100%

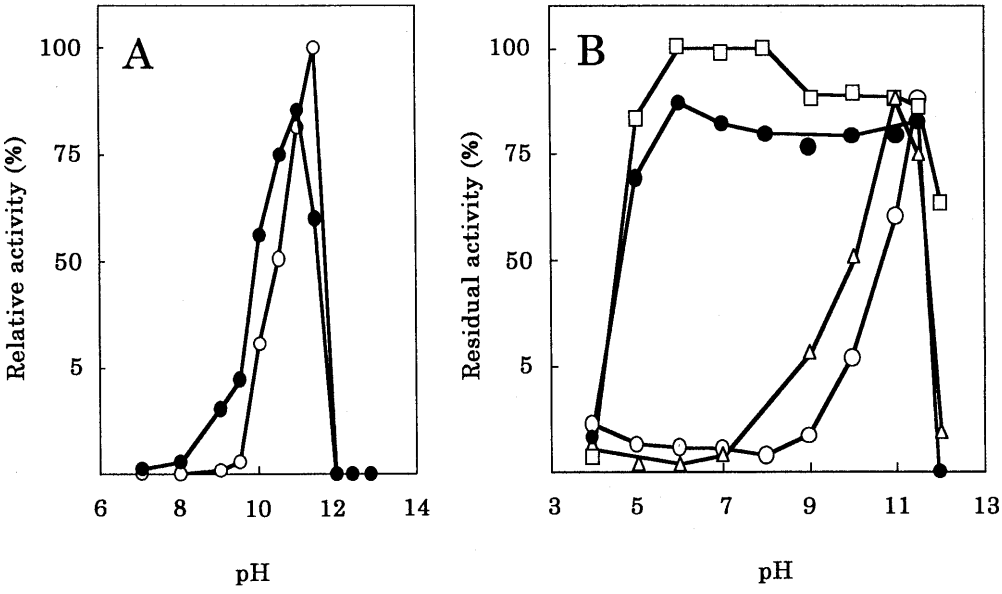

Fig. 2A,B. Effects of pH and NaCl on activity and stability. **A** Effect on activity. Pel activity was assayed at 30°C and at the indicated pH either in 50 mM Tris-HCl buffer (pH 7–9.5), 50 mM glycine-NaOH buffer (pH 8–12), or in 50 mM KCl-NaOH buffer (pH 12–12.8), with 0.03 μg Pel-4A. *Open circles*, activity without additive; *solid circles*, activity with 100 mM NaCl. **B** Effect on stability. Pel-4A (0.4 μg) was incubated for 60 h at 5°C and at the indicated pH in 50 mM each of various buffers in the absence (*open circles*) or presence (*solid circles*) of 100 mM NaCl, 2.0 mM $CaCl_2$ (*open triangles*), or both additives (*open squares*). Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity at pH 10.5 (without preincubation) was taken as 100%. Buffers used: acetate, pH 4–6; MOPS, pH 6–8; Tris-HCl, pH 7–9; glycine-NaOH, pH 9–12

lization by NaCl (100 mM) was observed at a pH range from 5 to 11.5, whereas the enzyme was stable only around pH 11 in the absence of NaCl (Fig. 2B). Calcium ions enhanced the pH stability of Pel-4A additionally and marginally. These results suggest that NaCl contributes appreciably to both activation and stabilization of Pel-4A. Moreover, the specific activity of the activated enzyme was as high as 8100 units $(mg\,protein)^{-1}$ under the optimal conditions at 60°C and at pH 11.0, a value that is the highest among Pels reported to date.

Nucleotide and deduced amino acid sequences

The nt sequence of the *pel*-4A gene cloned in the recombinant plasmid, extending from the 5′-terminal *Bam*HI site at nt 1 to the 3′-terminus at nt 1959, was analyzed (Fig. 3).

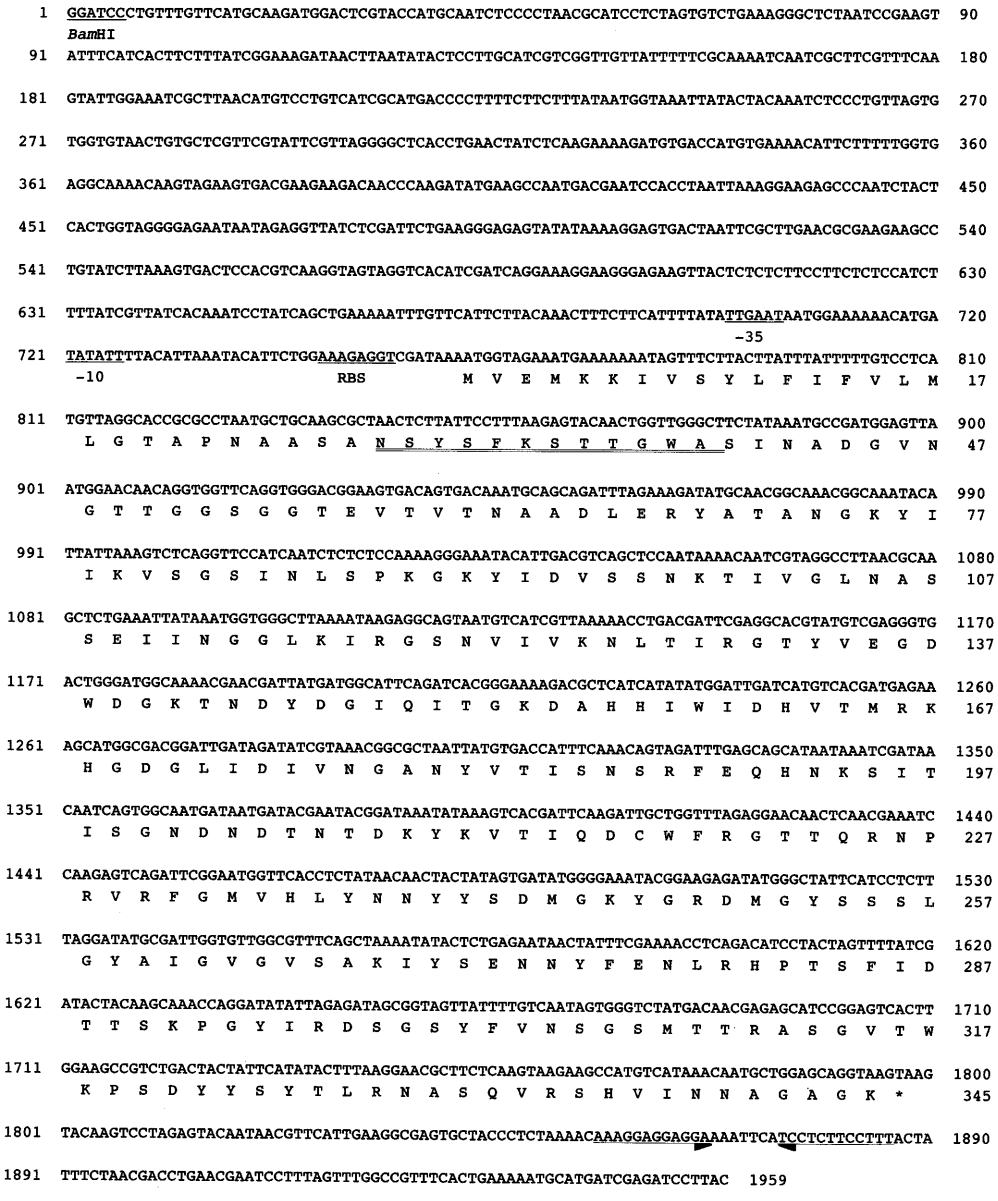

Fig. 3. Nucleotide sequence and the deduced amino acid sequence of the entire *pel*-4A gene and its flanking regions. *Numbers* on either side of the sequences denote nt and aa positions, respectively. The nt sequence similar to –35 and –10 consensus promoters of *B. subtilis* is *underlined*. The possible ribosome-binding site is indicated by *RBS*. The *pel*-4A ORF extends from Met1 to Lys345. The *double-underlined* aa sequence (Asn28–Ala39) refers to the N-terminal end of the extracellular, mature enzyme produced by *B. subtilis*. Inverted repeats downstream of the stop codon TAA (*) are indicated by *convergent arrows*

Starting from an ATG initiation codon at nt 762, there was a long open reading frame (ORF) of 1038 bp that ended in a TAA stop codon at nt 1799. The G + C content of the ORF was 39.7%. The putative ribosome-binding site, with the sequence 5′-AAAGAGGT-3′, was found 8 bp upstream of this ORF. The sequence of the ribosome-binding site had a free energy value of –46.5 kJ mol^{-1}. There was a putative sequence of sigma A-type promoter of *B. subtilis* (Moran et al. 1982), with 5′-TTGAAT-3′ as the potential –35 region and 5′-TATATT-3′ as the potential –10 region, separated by 16 bp. A long inverted-repeat sequence from nt 1856 to 1886 was found 57 bp downstream of the termination codon. The free energy value for a stem-loop structure was calculated to be –82.1 kJ mol^{-1}, which is sufficient for transcription termination and formation of a mRNA hairpin loop. The loop was followed by some T-residues, suggesting a *Rho*-independent type of transcription termination. The ORF encoded 345 aa including a putative signal sequence of 27 aa, as shown under the nt sequence in Fig. 3. The aa sequence deduced from the nt sequence of the *pel*-4A gene contained a hydrophilic/hydrophobic sequence from Met1 to Ala21 that is similar to signal peptides of *B. subtilis* (Simonen and Palva 1993). A deduced aa sequence identical to the 12 N-terminal Asn to Ala of the recombinant Pel-4A was found at aa 28–39. If the putative signal peptide Ala-Ser-Ala (aa 25–27) were cleaved on the C-terminal side of Ala27, the calculated molecular mass of mature Pel-4A (318 aa; Asn28–Lys345) would be 34,805 Da, a value very close to the 34 kDa determined for the recombinant Pel-4A.

Amino acid homology and possible catalytic residues

When suitably aligned, the deduced amino acid sequence of mature Pel-4A was found to exhibit some homology to

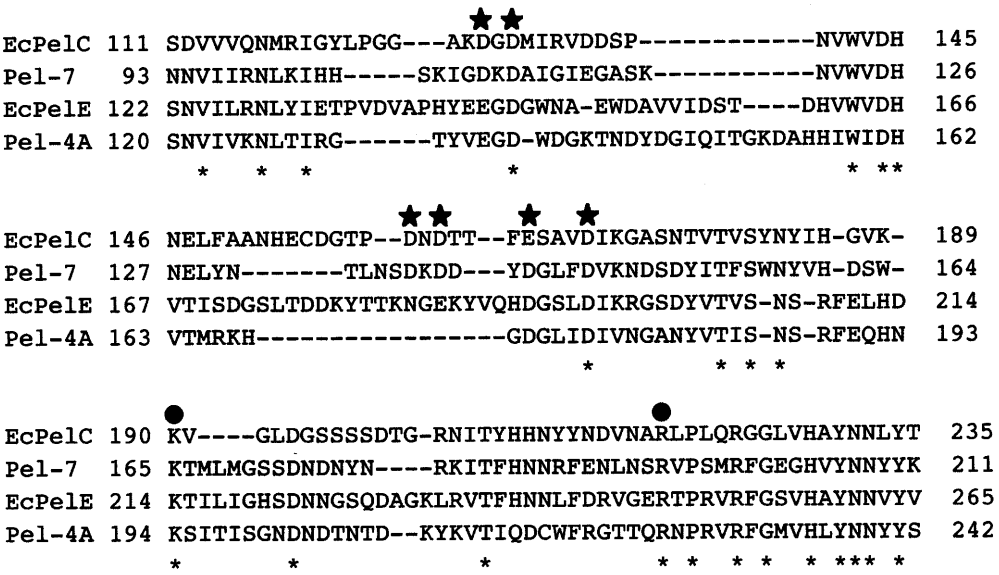

Fig. 4. A multiple alignment of the deduced amino acid sequences of Pel-4A with those of other Pels in the polysaccharide lyase family 1. The aa sequence alignment was done with the GENETYX program (SDC Software Development, Tokyo, Japan). The putative catalytic residues (*circles*) and calcium- and/or substrate-binding site (*stars*) proposed for PelC from *Erwinia chrysanthemi* EC16 (Scavetta et al. 1999) are shown above the sequence. Sequence sources aligned: EcPelC, *E. chrysanthemi* EC16; Pel-7, *Bacillus* sp. strain KSM-P7; EcPelE, *E. chrysanthemi* 3937; Pel-4A, *Bacillus* sp. strain P-4-N (this study)

those of known Pels. Pel-4A shared 32.1%–35.4% overall identity with reported Pels in the polysaccharide lyase family 1, such as PelA, PelD, and PelE from *E. chrysanthemi* EC16 (Keen and Tamaki 1986; Tamaki et al. 1988), PelE from *E. chrysanthemi* 3937 (Reverchon et al. 1989), Pel-7 from *Bacillus* sp. strain KSM-P7 (Kobayashi et al. 1999), and BsPel from *B. subtilis* SO113 (Nasser et al. 1993). It showed slightly higher homology to a Pel from *Pseudomonas viridiflava* (Liao et al. 1992) with 38.0% identity, whereas it had lower identity with the representative PelC from *E. chrysanthemi* EC16 (Tamaki et al. 1988) with 27.2% identity.

The deduced aa sequence of Pel-4A was suitably aligned with those of PelC from *E. chrysanthemi* EC16, PelE from *E. chrysanthemi* 3937, and Pel-7 from *Bacillus* sp. stain KSM-P7, all of which are in the polysaccharide lyase family 1 (Fig. 4). Lys190, Arg218, and Arg223 are suggested to form catalytic residues in the *Erwinia* PelC after site-directed mutagenesis (Kita et al. 1996). The putative catalytic residues are also proposed to be Arg279 or Arg264 in BsPel (Pickersgill et al. 1994) and Lys107, Lys129, and Arg132 in Pel-15 (Hatada et al. 2000). Recently, Scavetta et al. (1999) identified Lys190 and Arg218 for catalysis and Asp129, Asp131, Asp160, Asp162, Glu166, and Asp170 for substrate or calcium binding in PelC by the crystallographic analysis of an enzyme–plant cell wall complex. Therefore, Glu135, Asp137, Asp170, Asp174, Lys194, and Arg225 are likely to play indispensable roles in the *trans*-eliminative cleavage by Pel-4A, despite the limited overall-sequence identity and difference in aa usage among the Pels selected for alignment.

The catalytic properties of Pel-4A are significantly different from those of other Pels so far reported. Pel-4A is very unusual in that it has a very high specific activity and is activated and stabilized by excess NaCl. Recently, Tardy et al. (1997) reported that PelE from *E. chrysanthemi* 3937 is also activated by 50 mM NaCl in 100 mM Tris-HCl buffer (pH 8.5), which shows 34.6% identity with Pel-4A. The aa similarity of both enzymes is as high as 68.3%. The comparison of the aa sequences in Fig. 4 cannot account for the activation and stabilization by NaCl of Pel-4A, but it appears that excess salt is somehow involved in the catalytic step or changes the structural integrity and rigidity of the enzyme.

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