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Pectate lyase from *Bacillus subtilis*: molecular characterization of the gene, and properties of the cloned enzyme

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Pectate lyases (PL) initiate soft-rot diseases in plants by cleaving pectin which is the major component of the plant cell wall. The present paper reports the first cloning and characterization of a pectate lyase (*pel*) gene from the *Bacillus* genus. This gene was isolated from a *Bacillus subtilis* genomic library constructed in pUC18 as vector and *Escherichia coli* as host. By Southern hybridization this gene was shown to be present in a single copy in the *B. subtilis* genome. The nucleotide sequence of a 1.6 kb-pair *Hin*dIII restriction fragment, which confers pectate lyase activity to *E. coli*, indicated a 1,260 bp open reading frame encoding a 420 amino acid polypeptide which includes a 21 amino acid signal sequence. The 45,605 Da deduced protein displays homologies to PLs from *Erwinia chrysanthemi*. The *B. subtilis* PL cloned in *E. coli* was located in the periplasm. It was purified to homogeneity in a one-step procedure from the *E. coli* periplasmic fluid after overproduction using the pT7 system. Biochemical properties of the purified enzyme were similar to those found for the PL isolated from *B. subtilis* extracellular media.

pel gene; Gene expression; Purification; *Bacillus subtilis*

1. INTRODUCTION

Pectate lyase (PL) (EC 4.2.2.2) cleaves the α-1,4 glycosidic bond of polygalacturonic acid and generates unsaturated oligogalacturonides [1]. This class of pectinases is widely distributed in bacteria and fungi [2–6], some being phytopathogenic and others, such as members of the genera, *Klebsiella* and *Yersinia* [7–9], being non-phytopathogenic. The most well-known pectinolytic bacteria are the phytopathogenic *Erwiniae* that are the causal agents of soft-rot disease of many plant species [10,11]. These bacteria produce multiple isoenzymes of PL that are responsible for plant tissue maceration [12]. Bacteria from the *Bacillus* genus also produce PL and have been shown to cause soft-rot disease under certain conditions [13–17]. This raises the question of whether *Bacillus* is an opportunistic bacteria or an actual pathogen. The PL enzyme from *B. subtilis* has been purified and characterized by Nasser et al. [4]. This enzyme immunologically cross-reacted with PelB and PelC, the two neutral PL isoenzymes from *E. chrysanthemi*. Moreover crystals of this *B. subtilis* enzyme have been obtained and preliminarily studied by X-rays [18]. Recently, crystallization and thus a model of the three-dimensional structure of PelC from *E. chrysanthemi* has also been achieved [19].

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Although PL activity was described in *Bacillus* a long time ago, no genetic characterization of a PL gene had been carried out up to now. In the present paper, we report the first isolation and sequencing of a gene encoding PL activity in *B. subtilis*. Comparison of *B. subtilis* and *E. chrysanthemi* PL reveals the existence of conserved motifs the biological significance of which is discussed. Moreover this work explores the N-terminal sequence of the mature enzyme and some biochemical and enzymatic properties of the enzyme over-produced in *E. coli*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids, media and growth conditions*

Bacterial strains and plasmids used in this study are listed in Table I.

B. subtilis and *E. coli* were usually grown at 37°C in liquid or solidified agar (15 g/l), LB medium or synthetic M63 minimal medium supplemented with glycerol (0.1%) [20]. When required, antibiotics, ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km) were added at 50 $\mu g \cdot ml^{-1}$. For *B. subtilis* genomic DNA preparation, the cells were grown as described by Rodriguez and Tait [21].

2.2. *PL activity assay*

B. subtilis PL assay, K_m, V_m and optimum pH values were determined as previously described by Nasser et al. [4].

2.3. *DNA manipulations*

DNA-modifying enzymes and restriction endonucleases were purchased from Boehringer-Mannheim.

Chromosomal and plasmid DNA preparations were carried out as described by Ausubel et al. [22]. DNA digestion and fragment isolation, dephosphorylation, ligation, electrophoresis and bacterial transformation were carried out according to Sambrook et al. [23]. DNA

fragment isolation was performed after digestion and electrophoresis on agarose gels, with Geneclean II kit (Bio 101 Inc.) or by electroelution, using Biotrap apparatus (Schleicher and Shüell).

For Southern blot hybridization, DNA probes (restriction fragments or oligonucleotides) were labelled by random priming or digoxigenin (DIG) 5′ end labelling, using Boehringer-Mannheim kits. Southern blot hybridizations were performed on nylon membrane (Hybond N^+, Amersham) as proposed by the manufacturer.

2.4. *Preparation of a gene library and in situ screening of PL activity*

Chromosomal DNA was extracted from *B. subtilis* strain SO113 and partially digested with the restriction endonuclease, *Sau*3A. The obtained random fragments were separated on agarose (0.5%) gels and fragments between 3–6 kb-pair (kbp) were recovered by electroelution, using the Biotrap (Schleicher and Shüell) apparatus. These fragments were ligated with the plasmid vector, pUC18 (Appligene), which had been digested to completion with the restriction endonuclease, *Bam*HI, and dephosphorylated. The library was used to transform *E. coli* NM522 cells and the recombinants were selected on agar plates containing Ap. Recombinant clones were screened for PL activity as described by Keen et al. [24].

2.5. *Nucleotide sequence and computer analysis*

For nucleotide sequence analysis, a nested series of deletion clones was created using various restriction endonucleases. Sequencing was done using the chain termination method on double-stranded DNA templates. Extention of primers (M13 primer or M13 reverse primer) was carried out with T7 DNA polymerase (T7 sequencing kit from Pharmacia).

The resulting data were analyzed using the Mac Molly programme (Soft Gene, Berlin). Amino acid sequence comparisons were achieved with the CLUSTAL program.

2.6. *Over-production and purification of the* B. subtilis *PL in* E. coli

The 1.6 kbp *Hin*dIII–*Hin*dIII DNA fragment encoding the *B. subtilis* PL was cloned into pT7-5 and pT7-6 expression vectors leading to plasmids pNP111 and pNP112 (Table I). Expression of these resulting plasmids was performed in the presence of L-[^{35}S]methionine and cysteine in order to selectively label the *pel* gene product [25].

The periplasmic fluid, which contained PL activity, was released by osmotic shock as described by Nossal and Heppel [26]. The final concentration of this extract was adjusted to 20 mM Tris-HCl, pH 7, 1 mM ethylenediaminetetraacetique (EDTA) and 1 mM dithiothreitol (DTT) (extraction buffer). This preparation (50 ml) was applied to a Protein-Pack SP 8HR (1 × 10 cm) (Waters) column previously equilibrated with the extraction buffer. The column was washed with the same buffer and the proteins were eluted at 1.4 ml/min with a gradient from 0 to 0.6 M NaCl, using a Waters HPLC system. Fractions of 0.7 ml were collected.

2.7. *Analytical methods and other techniques*

Protein concentration determination was carried out as proposed by Bradford [27] using the Bio-Rad protein assay, with bovine serum albumin as a standard.

SDS-PAGE was performed according to Laemmli [28] on slab gels (12% resolving gel and 4% stacking gel). Protein bands were detected by Coomassie blue staining. Molecular weight markers were obtained from Bethesda Research Laboratory.

N-Terminal amino acid sequence of the purified PL was determined by automated Edman degradation [29], using a gas-phase protein sequencer.

3. RESULTS AND DISCUSSION

3.1. *Cloning and characterization of the* B. subtilis *gene encoding PL activity*

The strategy used to isolate the *B. subtilis pel* gene was the selection of recombinant plasmids exhibiting PL activity in *E. coli*. Among 5,000 *E. coli* transformants, two clones showing PL activity were detected. Restriction mapping of the plasmids isolated from these two clones revealed that they contained overlapping inserts of 2.8 and 3.3 kbp. In addition, Southern blot experiments showed that both inserts specifically hybridized to the oligonucleotide probe deduced from the N-terminal amino acid sequence of the purified PL (data not shown). Further studies were performed on the clone with the longest insert, pNP1 (Fig. 1).

To determine the size of the *pel* gene, the plasmid pNP1 was digested with different restriction endonucleases and the resulting fragments were inserted into

Table I

Bacterial strains and plasmids

Strains and plasmids	Genotypes and characteristics[a]	Origin or reference
Strains		
Bacillus subtilis SO113	*trpC2, amy-3*	[40]
Escherichia coli NM522	*SupE, thi, Δ(lac-pro AB), Δhsd5,* ($r_{k-}m_{k-}$)[*F′,pro AB, lacI*^{q}*ZΔ*M15]	Stratagene
Escherichia coli K38	HfrC, λ^+, *phoA4, pit–10, tonA22, ompF627, relA1*	[41]
Plasmids		
pUC18	Ap^R	Appligene
pBluescript (pBS)	Cm^R, *lacZ′*	Stratagene
pT7-5	Ap^R, T7 ϕ10	[25]
pT7-6	Ap^R, T7 ϕ10	[25]
pGP1-2	Km^R, P_L-T7 gene *1*, P*lac-c*1857	[25]
pNP1	pUC18 with 3.3 kbp *Sau*3A–*Sau*3A fragment containing the *pel* gene of *B. subtilis* SO113	This work
pNP2	pUC18 with 2.8 kbp *Sau*3A–*Sau*3A fragment containing the *pel* gene of *B. subtilis* SO113	This work
pNP11	pBS with 1.6 kbp *Hin*dIII–*Hin*dIII fragment containing the *pel* gene of *B. subtilis* SO113	This work
pNP111	pT7-5 with the *Xba*I–*Cla*I fragment from pWNPL11 containing the *pel* gene	This work
pNP112	pT7-6 with the *Xba*I–*Cla*I fragment from pWNPL11 containing the *pel* gene	This work

[a] Genotype symbols are according to Bachmann [46]. *lacZ′* indicates that the 3′ end of this gene is truncated. Ap^R, resistance to ampicillin; Cm^R, resistance to chloramphenicol; Km^R, resistance to kanamycin.

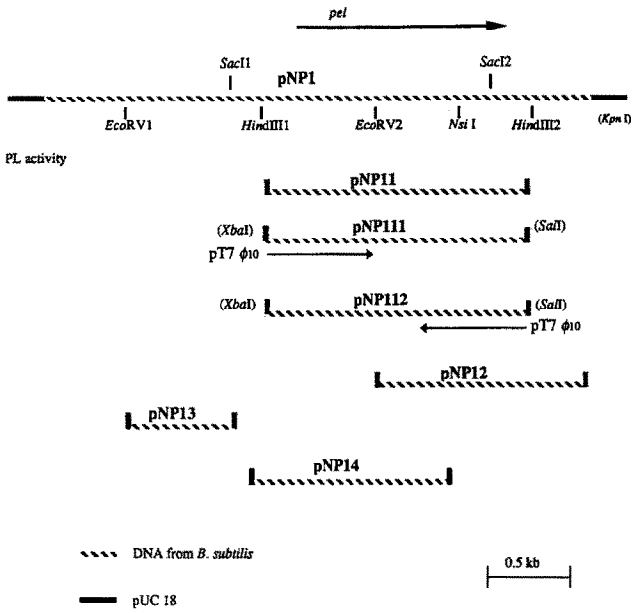

Fig. 1. Physical map of the 3.3 kbp *Sau*3A–*Sau*3A DNA fragment (pNP1) containing the *pel* gene from *B. subtilis* and subclone derivatives. The PL activity was checked as described by Nasser et al. [4]. pNP11 was constructed by insertion of the *Hin*dIII-*Hin*dIII fragment into the *Hin*dIII site of pBS; pNP12 by insertion of the *Eco*RV–*Kpn*I fragment into the *Eco*RV–*Kpn*I site of pBS; pNP13 by insertion of the *Eco*RV–*Sac*I fragment into the *Eco*RV–*Sac*I site of pBS; pNP14 by insertion of the *Hin*dIII–*Nsi*I fragment into the *Hin*dIII–*Pst*I site of pBS. Only *E. coli* harbouring pNP11 displayed PL activity. Restriction sites between brackets are sites from the vector polylinkers. The transcriptional direction of the *pel* gene, demonstrated by phage T7 expression system, is indicated by the arrow.

the appropriate sites of pBS, giving rise to plasmids pNP11, pNP12, pNP13 and pNP14 (Fig. 1). These plasmids were introduced into *E. coli* NM522 and checked for PL activity (Fig. 1). The smallest fragment exhibiting PL activity was the 1.6 kbp *Hin*dIII–*Hin*dIII fragment of pNP11. Cloning of this fragment in the two opposite orientations downstream of the pBS *lac* promoter had no effect on *pel* gene expression. This result indicated that the *pel* gene was probably expressed from its own promoter on the 1.6 kbp DNA fragment. To determine the *pel* gene transcriptional direction, the 1.6 kbp *Xba*I–*Sal*I restriction fragment from pNP11 was cloned into plasmids pT7-5 and pT7-6 digested with the same endonucleases (Fig. 1). These vectors differ by the orientation of the polylinker adjacent to the ϕ10 promoter which is specifically recognized by the T7 RNA polymerase. Expression of the genes present on the resulting plasmids, pNP111, pNP112, with specific labelling of the translated proteins, revealed two polypeptides of 42 kDa and 33 kDa produced from pNP111 (Fig. 2). The molecular weight of the first polypeptide is in accordance with the molecular weight of the mature PL protein from *B. subtilis*, previously determined by Nasser et al. [4]. The 33 kDa polypeptide might be a degradation product from the 42 kDa, as indicated by its positive immunological reaction by Western blot analysis (data not shown). Thus the labelling of the translated products from the T7 promoter-specific mRNAs demonstrated that the *pel* gene is transcribed in the *Hin*dIII–*Hin*dIII2 direction (Fig. 1).

Genomic *B. subtilis* DNA digested with *Eco*RV, *Eco*RI or *Hin*dIII was probed with the 1.6 kbp *Hin*dIII fragment from plasmid pNP11, containing the *pel* gene. Two different *Eco*RV fragments of 1.3 and 2 kbp hybridized with *pel* DNA. Two *Eco*RI fragments of 1.4 and 9 kbp were also detected and only one *Hin*dIII band of 1.6 kbp. This hybridization pattern correlates to the restriction map established for the *pel* gene. This suggests that the *pel* gene is present in a single copy on the *B. subtilis* genome.

3.2. *Purification and properties of the* B. subtilis *PL expressed in* E. coli

B. subtilis SO113 PL, expressed from pNP111, was purified with the procedure described in section 2.6. SDS-PAGE analysis of proteins contained in a major peak (not shown) revealed a single homogenous protein band with an apparent molecular weight of 42 kDa (Fig. 3). The purification procedure used allowed the purification to homogeneity in one chromatographic step of 3.2 mg of PL with a yield of 55%. The fact that the enzyme was over-expressed and located in the *E. coli* periplasm facilitated this purification.

In addition to the fact that the enzyme purified from *E. coli* has the same molecular weight as the one isolated

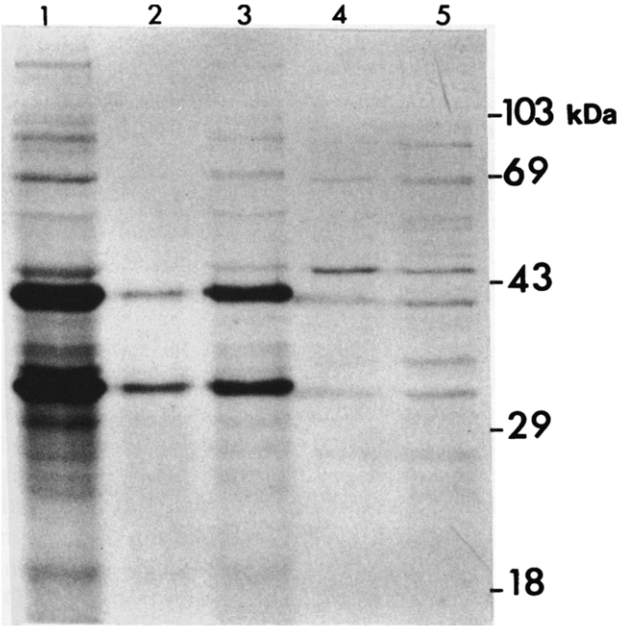

Fig. 2. Analysis of the *pel* gene product using the phage T7 promoter–polymerase expression system. After SDS-PAGE of the proteins synthesized in vivo by K38 (pGP1-2) carrying pNP111 (lane 1 and 3) (500,000 and 200,000 cpm, respectively), pNP112 (lane 2) (500,000 cpm), pT7-5 (lane 4) (500,000 cpm) and pT7-6 (lane 5) (500,000 cpm), the labelled polypeptides were detected by autoradiography. The size markers are indicated at the right.

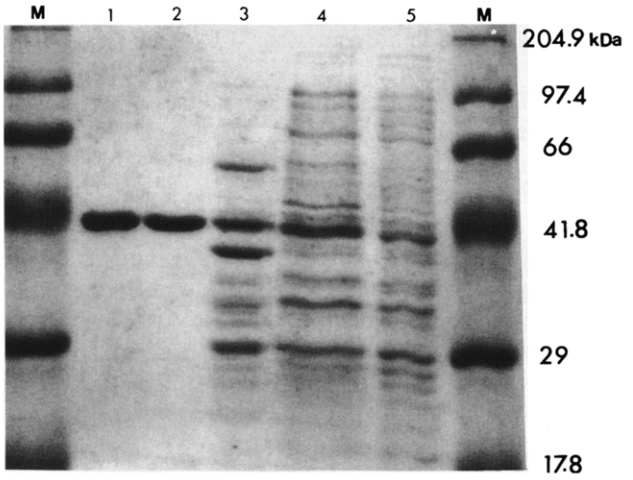

Fig. 3. Purification of the over-expressed *B. subtilis* PL. SDS-PAGE of 5 μg of purified PL from *B. subtilis* SO113 (lane 1); 5 μg of purified PL from *E. coli* K38 harbouring pNP111 (lane 2); 25 μg proteins from the periplasm of *E. coli* K38 harbouring pNP111 (lane 3); 40 μg of the crude extract from *E. coli* K38 harbouring pNP111 before the temperature shift to 42°C (lane 5) and after the temperature shift (lane 4). M lane denotes molecular weight markers.

from *B. subtilis*, they also have the same K_m and V_m values, exhibit maximum activity at the same pH, and display the same stability (data not shown and [4]). The influence of various enzyme inhibitors was tested on the purified PL. The enzyme was inhibited by hydroxymercuribenzoic acid and *N*-ethyl-maleimide which are SH enzyme inhibitors. The inhibition by the second compound was removed by the addition of DTT to the assay medium. These results suggest that a sulphydryl group may form a part of the catalytic site of the *B. subtilis* PL. The enzyme was also inhibited to a lesser degree by diethyl pyrocarbonate (DEPC) (inhibitor of tyrosine and histidine enzyme), whereas phenyl methyl sulfonyl fluoride (PMSF) (inhibitor of serine enzyme) had almost no effect on its activity. This suggests that the *B. subtilis* PL active site also contains a histidine or a tyrosine.

3.3. *Nucleotide sequence of the* pel *gene*

The complete nucleotide sequence of the 1.6 kbp *Hin*dIII–*Hin*dIII insert from pNP11 was determined on both strands, as mentioned in section 2.5. This sequence contains a single open reading frame (ORF) of 1,420 bp (Fig. 4), which starts with an ATG codon at nucleotide (nt) 205 and stops with a TAA termination codon at nt 1,464. The ORF encodes a polypeptide of 420 amino acids with a calculated molecular weight of 45,605 Da and a calculated pI of 7.9. The calculated molecular weight is slightly higher than that of the purified *B. subtilis* PL (42 kDa). This difference can be explained by the presence of a typical 21 amino acid signal peptide at the NH_2 extremity of the protein. The periplasmic localization of the cloned PL in *E. coli* indicates that this 21 amino acid signal peptide is functional in *E. coli* and is probably processed by the Sec system. The N-terminal amino acid sequence of the mature protein (Ala-Asp-Leu-Gly-His-Gln-Thr-Leu-Gly-Ser-Asn-Asp-Gly-Trp-Gly-Ala-Tyr-Ser-Thr-Gly-Thr-X-Gly-Gly-Ser-Lys-Ala), determined by Edman degradation, corresponds to that predicted from the nucleotide sequence of the *pel* gene and confirmed the signal sequence cleavage site. This signal peptide is sufficient to ensure extracellular localization of the PL in the Gram-positive bacterium *B. subtilis* [4]. In contrast, in Gram-negative bacteria, PLs are secreted into the extracellular medium using a two-step secretion pathway [30–33]. The first step, which is Sec-dependent, allows the passage of the protein though the inner bacterial membrane. This passage is accompanied by the cleavage of the peptide signal. The second step requires the Out proteins and allows the mature periplasmic enzyme to pass through the outer membrane. The fact that the *E. chrysanthemi* PLs cloned in *E. coli* remain localized in the periplasm [2,34] indicates that there is no functional Out machinery in *E. coli*.

Analysis of the nucleotide sequence upstream of the ATG initiation codon of the *B. subtilis pel* gene indicated the presence of a purine-rich sequence (AGAAAATGGGGGTA) that probably contains the ribosome binding site (RBS) [35,36]; it is worth noting that this sequence does not correspond to the well-defined Shine and Dalgarno sequence. Upstream from the putative RBS, there are putative −35 (TGAATG) and −10 (TATATT) promoter signals between nt 121 and nt 126 and between nt 144 and nt 150, respectively. It appears that the putative −10 region has good homology with the −10 promoter sequence recognized by the σ^{43} transcription factor of *B. subtilis* and by the σ^{70} transcription factor of *E. coli*. In contrast, the −35 region is not well conserved, suggesting that transcription of the *pel* gene may require another sigma factor or a specific positive regulatory protein.

Computer-aided searches revealed two inverted sequences (Fig. 4), one located upstream from the putative promoter sequence, between nt 22 and nt 59, and another downstream from the translation stop codon, between nt 1,475 and nt 1,508. The first inverted sequence may be involved in the transcription termination [37] of another gene located upstream from the *pel* gene, or it may also be the target site of a regulatory protein. Indeed a variety of regulators, such as products of *degQ, degR, degT, sacV, senN, senS,* and *tenA*, have been shown to stimulate the production of many extracellular enzymes in the genus *Bacillus* [38] by binding to sites generally located in the regulatory regions of the degradative enzyme genes. Analysis of the *B. subtilis pel* gene regulation would then be of great interest in order to define the biological significance of this inverted sequence. The second dyad symmetry sequence located 7 bp downstream from the TAA stop codon is able to form a secondary structure that may be involved in transcription termination [37] of the *B. subtilis pel* gene.

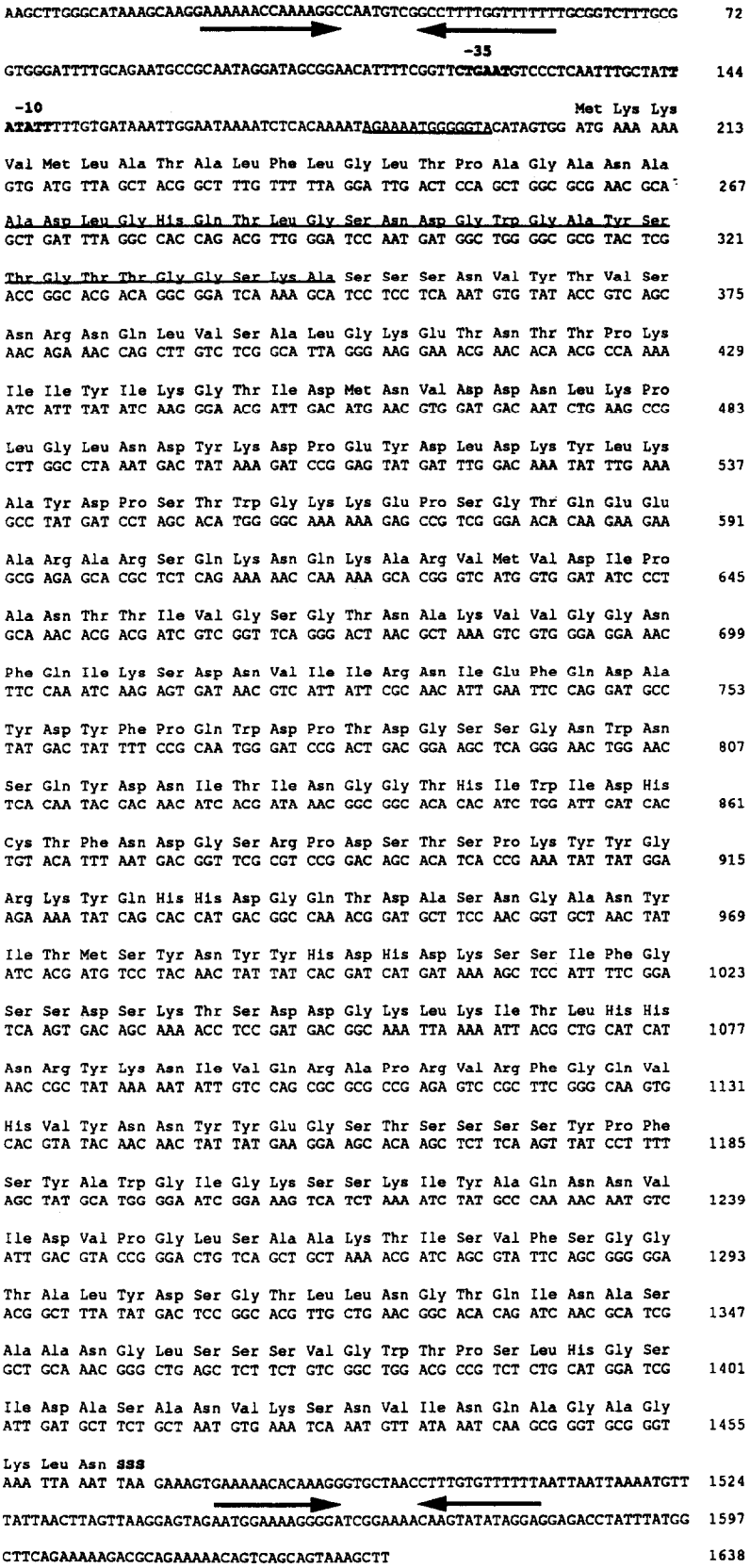

Fig. 4. Nucleotide sequence of the 1.6 kbp *Hin*dIII fragment including the *pel* gene. The putative promoter region (−35,−10) is in bold print. The peptidic sequence corresponding to the pel ORF is shown under the nucleotide sequence. The putative ribosome binding site and the N-terminal amino acid sequence of the mature protein, determined by Edman degradation, are underlined. Inverted repeat sequences are indicated by arrows. This sequence will appear in Genebank under the accession number: X74880.

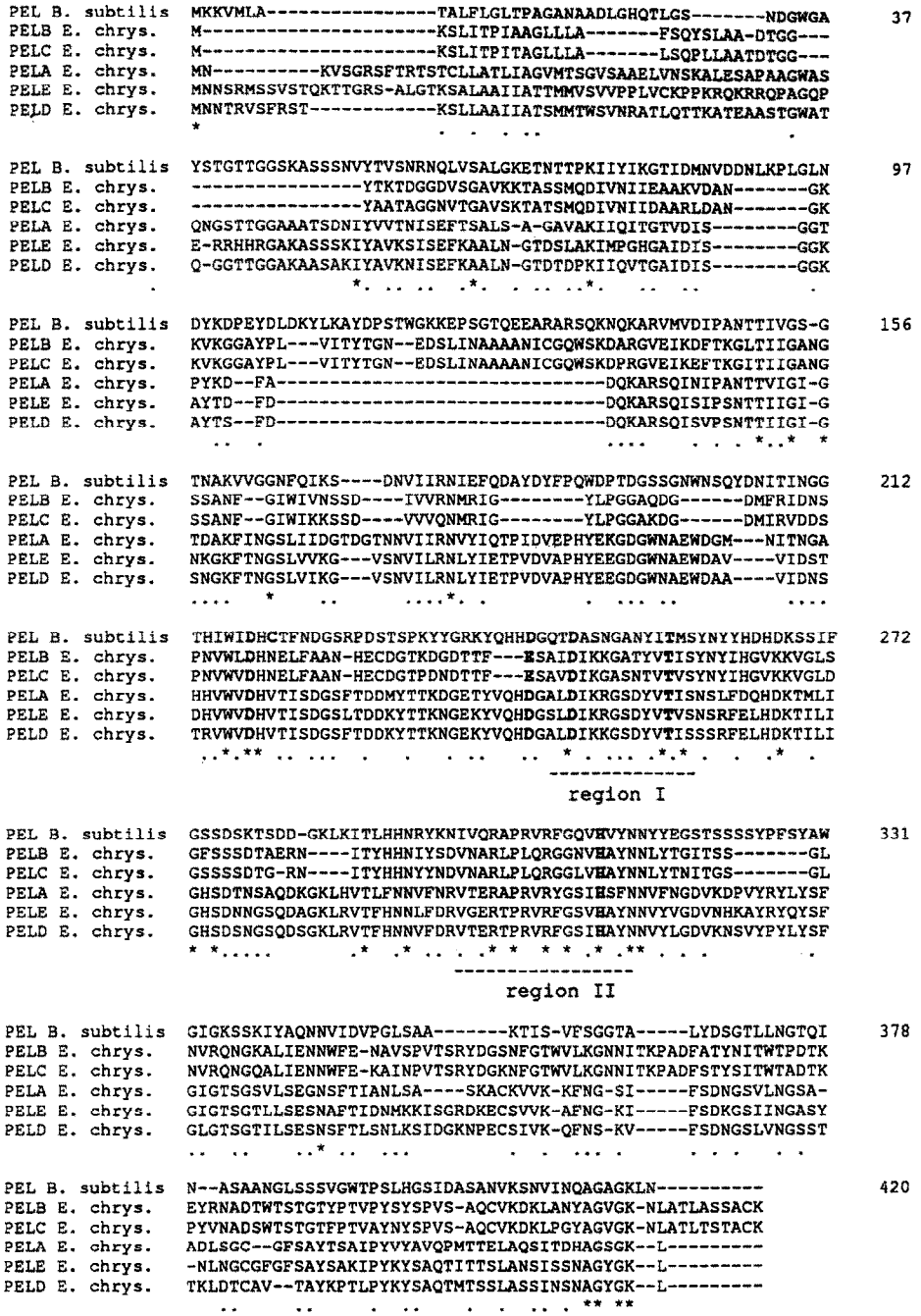

Fig. 5. Comparison of the *B. subtilis* PL with the five PL isoenzymes from *E. chrysanthemi* (data from PL sequences are from this study for *B. subtilis* PL, from Favey et al. [42] for 3937 PelA, from Van Gijsegem [43] for B374 PelD, from Reverchon et al. [44] for 3937 PelE and from Tamaki et al. [45] for EC16 PelB and PelC). Identical residues are indicated by asterisks; conservative substitutions are indicated by dots. Amino acids mentioned in the text (cysteine 220, aspartic acid 244 and 248, asparagine 210, tyrosine 255, and histidine 310) are in bold print. The two well-conserved regions in *E. chrysanthemi* PL isoenzymes spatially located around the putative Ca^{2+} binding site (Yoder et al. [19]) are underlined; amino acids 246–259 and amino acids 297–313 correspond, respectively, to region I and region II.

3.4. *Protein homology and structure*

The *B. subtilis* PL has only one cysteine in position 220, indicating that no disulphide bond is present in this enzyme. Moreover, specific inhibition of the *B. subtilis* PL by SH enzyme inhibitors demonstrated that this single cysteine residue may be involved in the catalytic site. The hydropathy profile, according to Kyte and Doolittle [39], of the deduced amino acid PL sequence showed that besides the region corresponding to the N-terminal signal peptide, which is hydrophobic, the mature enzyme is globally hydrophilic, as expected for a soluble protein.

Comparison of the *B. subtilis* PL amino acid sequence with those of the PLs from *E. chrysanthemi* revealed a globally weak homology. The homology decreases in the following order: PelA the acidic PL (34% identity), PelD and PelE, the two basic isoenzymes (31 and 30% identity, respectively) and PelB and PelC (26% identity) (Fig. 5). Although *B. subtilis* PL appears to be more similar to PelA, PelD, PelE than to PelB and PelC, this protein, purified either from *B. subtilis* (Nasser et al. [4]) or from *E. coli* (data not shown) is immunologically related to the *E. chrysanthemi* PelB and PelC and not to the three other isoenzymes. This result may be explained by a good conservation of sequences corresponding to antibody epitopes in these three proteins. This hypothesis is supported by the fact that the *B. subtilis* PL and *E. chrysanthemi* PelC isoenzymes present a very similar three-dimensional structure (Jenkins, personal communication). After crystallographic studies of the *E. chrysanthemi* PelC isoenzyme, Yoder et al. [19] demonstrated that this enzyme folds into a unique motif of parallel β-strands coiled into a large helix. Within the core, the amino acids form linear stacks and include a new asparagine ladder. It is interesting to note that asparagine residues are also highly conserved in the *B. subtilis* PL (Fig. 5). Moreover, since calcium is essential for PL activity, Yoder et al. [19] suggested the existence of a putative Ca^{2+} binding site involving the residues Asp-131, Glu-166 and Asp-170 in the *E. chrysanthemi* PelC isoenzyme. These acidic amino acids are conserved among all extracellular PLs, including the *B. subtilis* enzyme, where they correspond to Asp-210, Asp-244 and Asp-248, respectively. Further crystallographic analysis on the *B. subtilis* PL will be performed in the presence of Ca^{2+} to confirm this hypothesis. In addition, the two well-conserved regions that are spatially located around the putative Ca^{2+} binding site (Fig. 5), proposed by Yoder et al. [19], contains a conserved tyrosine (region I) and a conserved histidine (region II). These two residues may be involved in the catalytic site of these enzymes, as suggested by the DEPC inhibition. Site-directed mutagenesis will be performed in these regions to evaluate the importance of these two amino acids in the catalytic activity of PL.

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