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Molecular Cloning and Nucleotide Sequence of A Pectin Lyase Gene from Pseudomonas marginalis N6301

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SUMMARY : A pectin lyase (PNL;EC4.2.2.10) gene of Pseudomonas marginalis N6301 was cloned and expressed in Escherichia coli. We purified PNL from P.marginalis N6301 and determined N-terminal 33 amino acids sequence. From this sequence, we synthesized two oligonucleotide probes. From the analysis of Southern hybridization, 2.1kb EcoRI-SmaI fragment from the chromosomal DNA of P.marginalis was found to hybridize with oligonucleotide probes. Then, we cloned the fragment into pUC119 vector and transformed into E.coli DH5α. A plasmid thus obtained was designated as pPNL6301. E.coli DH5α harboring pPNL6301 expressed PNL activity. The nucleotide sequence of pnl gene in the plasmid pPNL6301 encoding PNL from P.marginalis N6301 was determined. The structural gene of pnl consisted of 936 base pairs. An open reading frame that encodes a 34,103 dalton polypeptide composed of 312 amino acids was assigned. The molecular weight of the polypeptide predicted from the amino acid composition was close to that of PNL of P.marginalis N6301 determined. The nucleotide sequence of the 5'-flanking region of pnl gene showed the presence of the consensus sequence of LexA binding site, Pribnow box and ribosome binding site as found in Escherichia coli. The amino acid sequence homology of PNLs and nucleotide sequence homology of pnl gene between P.marginalis N6301 and E.carotovora Er were 60.8% and 57.2%, respectively.

Pectolytic enzymes are often found in phytopathogenic microorganisms which are responsible for causing diseases in plants (1,2). Among pectinolytic enzymes of soft-rot-causing Erwinias, Erwinia carotovora and Erwinia chrysanthemi, pectin lyase (PNL) is a very unique enzyme because it was produced aboundantly only when bacterial cultures were exposed to nalidixic acid, mitomycin C, or UV light (3,4,5,6). We have recently found that another soft-rot bacterium, Pseudomonas marginalis N6301 also produced a massive amount of PNL when the culture was treated with mitomycin C. We also demonstrated that PNL from P.marginalis was different from that from E.carotovora in immunological properties (7). To study on the molecular mechanism of PNL production, we attempted to clone the pnl gene of E.carotovora and succeeded in cloning and determination of the complete nucleotide sequence of the pnl gene (8,9). In this report, cloning, expression and complete nucleotide sequence of the pnl gene of P.marginalis are described. Comparison of pnl gene of P.marginalis with that of E.carotovora was also described with special reference to the mechanism of the pnl gene expression.

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MATERIALS AND METHODS

Bacterial strains, phage and plasmid. E.coli DH5α [(F⁻recA1, endA1, gyrA96, thi-1, supE44, relA1, mcrA⁻ mcrB⁺, λ⁻, (argF-lacxya)U169, φ80dlacΔ15] was used as a host strain and a plasmid pUC119 was used as a vector for cloning. Plasmid pUC119 was used as vector and E.coli MV1184 [ara, Δ(lac-pro), strA, thi, (φ80, lacZ ΔZ ΔM15), (srl-recA)306::Tn10(tetʳ), F':traD36, proAB, lacIᵠ, Z ΔM15], and phage M13KO7 were used as a host, and a helper phage respectively, for preparation of single stranded DNA. Pseudomonas marginalis N6301 was used as a source of chromosomal DNA.

Preparation of mixed oligonucleotide probes for cloning of pnl gene. The N-terminal amino acids sequence of PNL was determined by Edman degradation method. Oligonucleotides were synthesized by a Applied Biosystems model 381A or a MilliGen/Bioserch, Cyclon™ Plus DNA synthesizer. Synthetic oligonucleotides were labeled with γ-^{32}P-ATP (>110TBq/mmol) as described by Maniatis et al (10).

Genomic Southern blot hybridization. About 1μg of purified DNA of P.marginalis N6301 was completely digested with a series of restriction enzymes and electrophoresed in 0.8% agarose gel. Fragments of DNA were transferred from the gel to nylon membrane filters and hybridized with the labeled probes. Hybridization with No.1 probe (Fig.1) was done at 42℃ for 18hrs in a solution containing 5xSSC (1xSSC: 0.15M NaCl, 0.015M sodium citrate), 5xDenhardt's reagent (1xDenhardt's reagent is 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.1%SDS, 10% formamide and 100ng/ml denatured salmon sperm DNA. Filters were washed twice (5 min. per wash) with 2xSSC-0.1%SDS at room temperature and once with 2xSSC-0.1%SDS at 42℃ for 10min. Hybridization with No.2 probe (Fig.1) was performed at 37℃ in the same solution as in the case of No.1 probe. Random primed DNA labeling kit (United States Biochemical) was used for labeling the 0.8kb PstI-EcoRI fragment.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al (13). A series of deletion derivatives of each subclone were obtained by exonuclease III and mung bean nuclease digestion according to the procedures described by Henikoff (16).

Preparation of cell extracts and assay of PNL. The cells of the plasmid-containing E.coli DH5α were grown at 37 ℃ in 2ml of LB medium containing ampicillin (100 μg/ml) without DNA damaging reagent. The cells were harvested by centrifugation at 10,000xg for 1 min. at 4 ℃. The cells were then suspended in 2ml of 10mM Tris-HCl buffer (pH7.5), sonicated and then centrifuged at 15,000xg for 1 min. at 4℃. The supernatant was used directly for PNL assay. PNL activity was determined by the method as described by Itoh et al (14). Protein content was determined by the method of Lowry et al (15).

RESULTS AND DISCUSSION

Analysis of N-terminal sequence of PNL. PNL from P.marginalis N6301 was purified to electrophoretic homogeneity from a cultural fluid (Manuscript in preparation). N-terminal amino acids of PNL were determined (Fig.1). According to the amino acid sequence, the oligonucleotide probes (No.1 and No.2) were synthesized (Fig.1).

Cloning of PNL gene in E.coli DH5α. Chromosomal DNA from P.marginalis N6301 was purified by the method of Rodriguez et al (11). Synthetic oligonucleotide probes were labeled with γ-^{32}P-ATP. Southern blot hybridization analysis was carried out with the fragments of chromosomal DNA of P.marginalis N6301 digested with several restriction enzymes using these labeled probes. We found 3.8kb PstI-PstI fragment was hybridized with both probes. Therefore, chromosomal DNA was digested with restriction enzyme PstI, fractionated by 0.8% agarose gel electrophoresis and 3.5-4.0kb fragments were extracted with GENECLEAN II DNA extraction kit. The extracted DNA was ligated into PstI site of pUC119 vector and transformed to E.coli DH5α. Four positive clones were obtained from about 2,000 recombinants. All four clones had 3.8kb

NH_2-SerTyrProGluSerLysLeuThrGlyLeuThrGlyPheAlaLeu

No.2-- AAGTTIACIGGITTIACIGGITTTGCITT

AlaAlaLysValThrGlyGlyTrpAlaGlyProValValSerIle

GCIGCIAAAGTIACIGGIGGITGGGCIGGICCIGTIGT--No.1
G

ThrAsnLeu-COOH

Fig. 1. N-terminal amino acid sequence of PNL and oligonucleotide probe sequences. Abbreviation: I; Inosine.

PstI-PstI fragment (Data not shown). The 0.8kb PstI-EcoRI region which was hybridized with No.1 probe was then sequenced. The nucleotide sequence, which corresponds to 33 amino acids of PNL was found in the 0.8kb fragment (Data not shown). Since 0.8kb fragment does not contain whole pnl gene, we performed further cloning, using 0.8kb fragment as a probe. The chromosomal DNA was digested with EcoRI and SmaI, fractionated by electrophoresis in a 0.8% agarose gel and 2.1kb fragment in size was extracted from agarose gel by GENECLEAN II DNA extraction kit. The extracted fragment was ligated to pUC119 DNA digested with EcoRI, SmaI and dephosphorylated. Approximately 200 colonies containing inserts (white colonies) were screened with 0.8kb fragment labeled with random primed DNA Labeling kit (United States Biochemical). Only one positive colony was obtained. Plasmid DNA from the positive colony containing 2.1kb EcoRI-SmaI insert was found to hybridize with the 0.8kb probe strongly. This plasmid was designated as pPNL6301. Physical map of the plasmid is shown in Fig.2.

Nucleotide sequence of the pnl gene from pPNL6301. We have determined the nucleotide sequence of the EcoRI-SmaI fragment containing pnl gene. The sequencing strategy is shown in Fig.2. The nucleotide sequence of the fragment was comprised of 2160bp (Fig.3). Within this sequence, we can identify an open reading frame which begins with an ATG codon at position 515 and terminates with TGA codon at position 1451. This 936 nucleotides encode 312 amino acids, and we deduced its molecular weight as 34,103 dalton. The N-terminal deduced 33 amino acids sequence exactly corresponded to that of purified PNL (Fig.3), and the initiating formyl methionine was removed posttranslationally. There was no indication of processing of N-terminus.

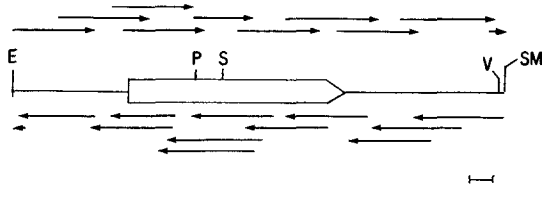

Fig. 2. Physical map and sequencing strategy of the 2160bp EcoRI-SmaI fragment. Thick bar indicates open reading frame. Arrows indicate the direction and sequence determined region of deleted DNA clones. Bar indicates 100bp. Restriction sites: E, EcoRI P, PstI S, SalI V, EcoRV SM, SmaI.

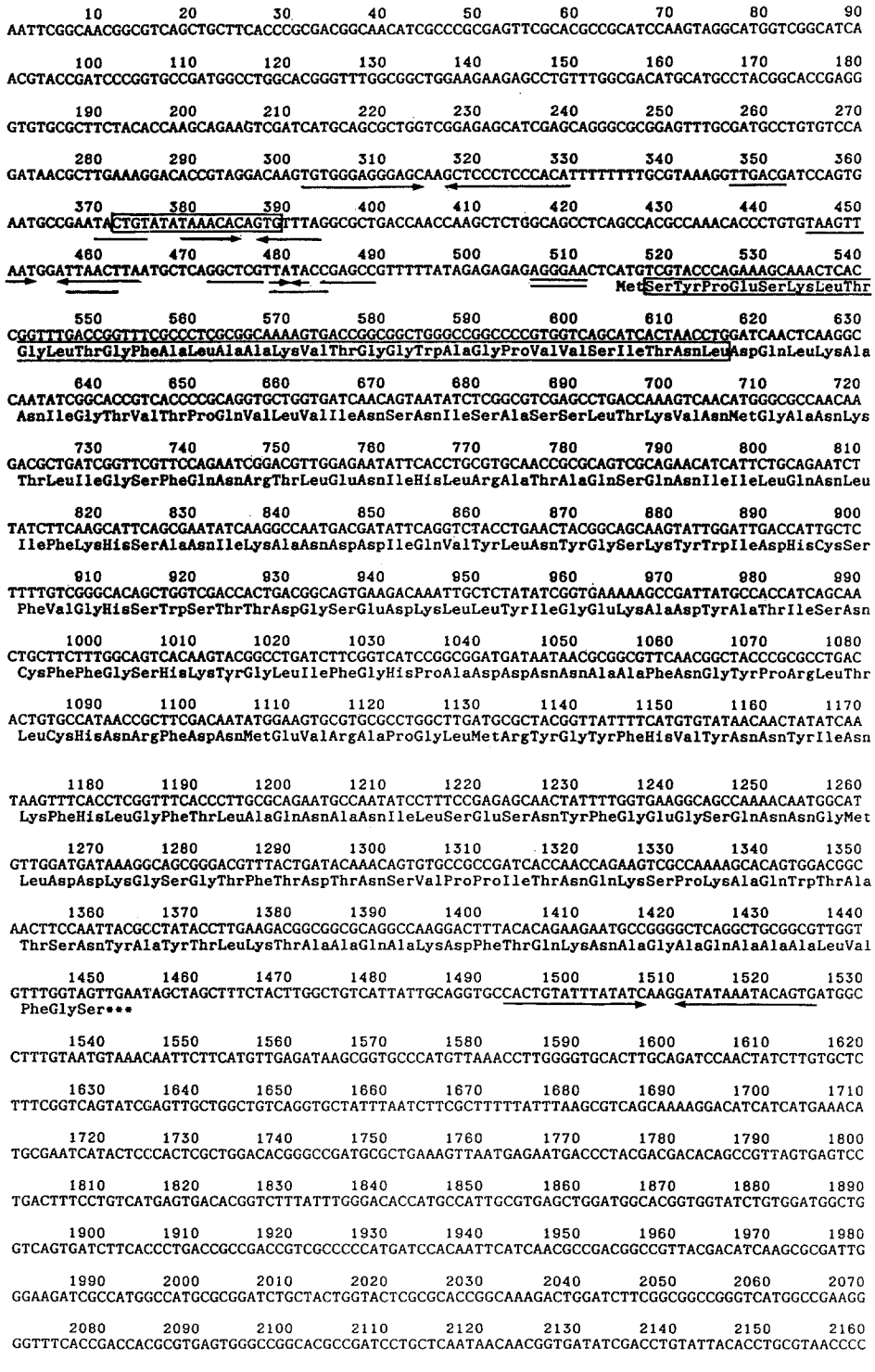

Fig.3. Nucleotide sequence of the EcoRI-SmaI fragment and the deduced amino acid sequence of pectin lyase (PNL). The strand shown is the 5' to 3' direction. The deduced amino acids sequence is given below the corresponding nucleotide sequence. The predicted ribosomal binding site and promoter sites are indicated by a double underline and single underlines, respectively. The nucleotide sequence of LexA binding site and N-terminal 33 amino acid sequence which determined from protein are boxed. Arrows represent inverted repeats.

Protein	Position	Sequence
P.marginalis	1- 60	MSYPESKLTGLTGFALAAKVTGGWAGPVVSITNLDQLKANIGTVTPQVLVINSNISASSL
E.carotovora	1- 60	MAYPTTNLTGLIGFAKAAKVTGGTGGKVVTVNSLADFKSAVSGSAKTIVVLGSSLKTSAL
P.marginalis	61-119	TKVNMGANKTLIGSF-QNRTLENIHLRATAQSQNIILQNLIFKHSANIKANDDIQVYLNY
E.carotovora	61-120	TKVVFGSNKTIVGSFGGANVLTNIHLRAESNSSNVIFQNLVFKHDVAIKDNDDIQLYLNY
P.marginalis	120-179	GSKYWIDHCSFVGHSWSTTDGSEDKLLYIGEKADYATISNCFFGSHKYGLIFGHPADDNN
E.carotovora	121-180	GKGYWVDHCSWPGHTWSDNDGSLDKLIYIGEKADYITISNCLFSNHKYGCIFGHPADDNN
P.marginalis	180-239	AAFNGYPRLTLCHNRFDNMEVRAPGLMRYGYFHVYNNYINKFHLGFTLAQNANILSESNY
E.carotovora	181-240	SAYNGYPRLTICHNYYENIQVRAPGLMRYGYFHVFNNYVNKFQLAFTVAQNANVISERNV
P.marginalis	240-298	FGEGSQNNGMLDDKGSG-TFTDTNSVPPITNQKSPKAQWTATSNYAYTLKTAAQAKDFTQ
E.carotovora	241-300	FGSGAEKKGMVDDKGNGSTFTDNGSSPAAVASKSPAAKWTASSNYSYSLMTTAAAQSWVV
P.marginalis	299-312	KNAGAQAAALVFGS
E.carotovora	301-314	SNAGAQNSALKFPS

Fig.4. Comparison of protein sequence of PNL from P.marginalis N6301(upper sequence) with that of E.carotovora Er(lower sequence). Vertical bars indicate the homology of bases.

Codon usage. The codon usage for the pnl gene was computed and the following biases were observed. Generally, the codon usage corresponds to that of genus Pseudomonas (17,18). But the following exception was found. (a) In the Glu codons, GAA is used more than GAG and AAA codon is used by 7 out of 18 Lys residues (11.3% is used in Pseudomonas(17)). (b) In the Phe, Tyr, His, Asn and Thr codon usages, tendency is very similar to those of Erwinia carotovora Er rather than those of genus Pseudomonas.

Amino acid homology among the PNLs. The nucleotide sequence of pnl gene and the deduced amino acid sequence of PNLs were determined in bacteria and fungi (9,19,20). Amino acid homology between P.marginalis N6301 and E.carotovora Er is shown in Fig.4. Total homology is 60.8%. Some amino acids difference is observed in the N-terminal and C-terminal regions, but a very high similarity is found in the center regions. The PNL from P.marginalis N6301 is very similar to those from E.carotovora Er (9) and E.carotovora subsp. carotovora 71 (19) but not from A.niger (20) in the amino acid sequence (9).

The nucleotide sequence of the 5'- and 3'- flanking regions. A potential Shine-Dalgarno sequence (AGGGAA) was properly positioned in 5' region of the putative ATG start codon (Fig.3). Several E.coli sigma 70-like promoter sequences are found in 5' region of this putative ribosome binding site. One of them, TTGACG(17bp)TACTGT, is found from positions 347 to 375. The LexA binding site is found from positions 372 to 389. This sequence corresponds to consensus sequence of LexA binding site of SOS genes (21). In both upstream and downstream the region (positions 347-375), we can find four inverted repeat sequences (positions 302-329,379-393,445-464 and 472-489). And we can also see another sequence which will be a

candidate for promoter site of -35 (positions 457-462) and -10 (positions 479-484) in this region. These data suggest that the expression of the pnl gene in P.marginalis N6301 is controled by not only LexA-RecA interaction (SOS response) but also another unknown protein interaction. In this connection, our previous observations in E.carotovora should be noted; we showed that there is no LexA binding site in upstream the translational start codon of pnl gene in E.carotovora, irrespective of recA-dependent induction of the PNL (9). Further experiment needs to support the hypothesis. Only one but long inverted repeat sequence (positions 1493-1525) found downstream the open reading frame of pnl gene, seems to be transcription termination signal.

Expression of pectin lyase activity in E.coli. The crude extract of E.coli DH5α harboring pPNL6301 or pUC119 (a control) was prepared and the PNL activity was determined. PNL activity (0.57unit per milligram protein) was detected only in E.coli DH5α harboring pPNL6301. The expression of pnl gene in E.coli DH5α which is recA1 mutant might be due to the gene dosage of the plasmid in the cells, or due to the use of the promoter (positions 457-462 and 479-484) which locates downstream the LexA binding site for synthesizing the mRNA.

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