

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

Naoki Nikaidou, Yoshiyuki Kamio and Kazuo Izaki*

Department of Agricultural Chemistry, Faculty of Agriculture,
Tohoku University, Amamiya-machi, Sendai 981, Japan

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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 DH5 α . A plasmid thus obtained was designated as pPNL6301. E. coli DH5 α 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. © 1992 Academic Press, Inc.

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 abundantly 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.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, phage and plasmid. *E. coli* DH5 α [(F⁻recA1, endA1, gyrA96, thi-1, supE44, relA1, mcrA⁻ mcrB⁺, λ ⁻, (argF-lacxya)U169, ϕ 80dlac Δ 15] 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, (ϕ 80, lacZ Δ Z Δ M15), (srl-recA)306::Tn10(tet^r), F':traD36, proAB, lacI^q, Z Δ M15], and phage M13K07 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 -³²P-ATP (>110TBq/mmol) as described by Maniatis *et al* (10).

Genomic Southern blot hybridization. About 1 μ g 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°C 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°C for 10min. Hybridization with No.2 probe (Fig.1) was performed at 37°C 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* DH5 α were grown at 37 °C in 2ml of LB medium containing ampicillin (100 μ g/ml) without DNA damaging reagent. The cells were harvested by centrifugation at 10,000xg for 1 min. at 4 °C. 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°C. 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 DH5 α . Chromosomal DNA from *P. marginalis* N6301 was purified by the method of Rodriguez *et al* (11). Synthetic oligonucleotide probes were labeled with γ -³²P-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* DH5 α . Four positive clones were obtained from about 2,000 recombinants. All four clones had 3.8kb

NH₂-SerTyrProGluSerLysLeuThrGlyLeuThrGlyPheAlaLeu

No.2-- AAGTTIACIGGITTACIGGITTGCITT

AlaAlaLysValThrGlyGlyTrpAlaGlyProValValSerIle

GCIGCIAAAGTIACIGGIGGTGGGICGGICCTGTGT--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 GENECLAN 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.

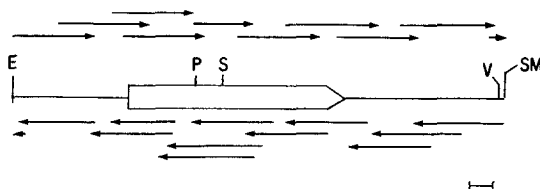


Fig.2. Physical map and sequencing strategy of the 2160bp EcoRI-SmaI fragment. Thick bar indicates open reading frame. Arrows indicate the direction and sequence determined region of deleted DNA clones. Bar indicates 100bp. Restriction sites: E, EcoRI P, PstI S, SalI V, EcoRV SM, SmaI.

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10      20      30      40      50      60      70      80      90
AATTCGGCAACGGCGTACGTGCTTACCCGCGACGGCAACATCGCCGCGAGTTCGCACGCCGCATCCAAGTAGGCATGGTCGGCATCA

100     110     120     130     140     150     160     170     180
ACGTACCGATCCCGGTGCCGATGGCCTGGCCACGGGTTTGGCGGCTGGAAGAAGACCTGTTTGGCGACATGCATCCACGGCACCAGG

190     200     210     220     230     240     250     260     270
GTGTGGCGCTTCTACACCAAGCAGAAGTCGATCATGCAGCGCTGGTCGGAGAGCATCGAGCAGGGCGCGAGTTTGGCATGCCCTGTGTCCA

280     290     300     310     320     330     340     350     360
GATAACGCTTGAAGGACACCGTAGGACAAGTGTGGGAGGAGCAAGTCCCTCCCACATTTTTTGGCTAAAGGTTGACGATCCAGTG

370     380     390     400     410     420     430     440     450
AATGCCGAATACTGTATATAAACACAGTGTAGGCGCTGACCAACCAAGCTCTGGCAGCCTCAGCCACGCCAAACCCCTGTGTAAGTT

460     470     480     490     500     510     520     530     540
AATGGATTAACTAATGCTCAGGCTCGTTATACCGAGCCGTTTTTATAGAGAGAGGGAACATCATGTCGTACCCAGAAAGCAAATCAC
MetSerTyrProGluSerLysLeuThr

550     560     570     580     590     600     610     620     630
CGGTTTGACCGGTTTCGCCCTCGCGGCAAAAGTGACCGCGGCTGGGCGGCGCCCGTGGTCAGCATCACTAACCTGGATCAACTCAAGGC
GlyLeuThrGlyPheAlaLeuAlaAlaLysValThrGlyGlyTrpAlaGlyProValValSerIleThrAsnLeuAspGlnLeuLysAla

640     650     660     670     680     690     700     710     720
CAATATCGGCACCGTCACCGCGAGGTGCTGGTATCAACAGTAATATCTCGGCGTCGAGCCTGACCAAAGTCAACATGGCGCCCAACAA
AsnIleGlyThrValThrProGlnValLeuValIleAsnSerAsnIleSerAlaSerSerLeuThrLysValAsnMetGlyAlaAsnLys

730     740     750     760     770     780     790     800     810
GACGCTGATCGGTTCTGTCAGAATCGGACGTTGGAGAATATTCACTCGCTGCAACCGCGCAGTCGCAGAACATCATTTCTGCAGAATCT
ThrLeuIleGlySerPheGlnAsnArgThrLeuGluAsnIleHisLeuArgAlaThrAlaGlnSerGlnAsnIleIleLeuGlnAsnLeu

820     830     840     850     860     870     880     890     900
TATCTTCAACGATTACGCGAATATCAAGGCCAATGACGATATTCAAGTCTACCTGAACTACGGCAGCAAGTATTGGATTGACCATTTGCTC
IlePheLysHisSerAlaAsnIleLysAlaAsnAspAspIleGlnValTyrLeuAsnTyrGlySerLysTyrTrpIleAspHisCysSer

910     920     930     940     950     960     970     980     990
TTTGTGCGGCACAGCTGGTCGACCACTGACGGCAGTGAAGACAAATTTGCTCTATATCGGTGAAAAAGCCGATTATGCCACCATCAGCAA
PheValGlyHisSerTrpSerThrThrAspGlySerGluAspLysLeuLeuTyrIleGlyGluLysAlaAspTyrAlaThrIleSerAsn

1000    1010    1020    1030    1040    1050    1060    1070    1080
CTGCTTCTTTGGCAGTCACAAGTACGGCCTGATCTTCGGTCATCGCGCGGATGATAATAACGCGCGCTTCAACGGCTACCCGCGCTGAC
CysPhePheGlySerHisLysTyrGlyLeuIlePheGlyHisProAlaAspAspAsnAlaAlaPheAsnGlyTyrProArgLeuThr

1090    1100    1110    1120    1130    1140    1150    1160    1170
ACTGTGCCATAACCGCTTCGACAATATGGAAGTGGCTGCGCTGGCTTGATGCGCTACGGTTATTTTCATGTGTATACAACTATATCAA
LeuCysHisAsnArgPheAspAsnMetGluValArgAlaProGlyLeuMetArgTyrGlyTyrPheHisValTyrAsnAsnTyrIleAsn

1180    1190    1200    1210    1220    1230    1240    1250    1260
TAAGTTTACCTCGGTTTACCCTTGGCGAGAATGCCAATATCTTTCCGAGAGCAACTATTTTGGTGAAGCAGCCAAAACAATGGCAT
LysPheHisLeuGlyPheThrLeuAlaGlnAsnAlaAsnIleLeuSerGluSerAsnTyrPheGlyGluGlySerGlnAsnAsnGlyMet

1270    1280    1290    1300    1310    1320    1330    1340    1350
GTTGGATGATAAAGGCAGCGGACGTTTACTGATACAAACAGTGTGCCGCGGATCACCAACCAGAAGTCGCCAAAGCACAGTGGACGGC
LeuAspAspLysGlySerGlyThrPheThrAspThrAsnSerValProProIleThrAsnGlnLysSerProLysAlaGlnTrpThrAla

1360    1370    1380    1390    1400    1410    1420    1430    1440
AACTTCCAATTACGCTTACCTTGAAGACGGCGCGGCGCAAGGACTTTACACAGAAGAATGCCGGGGCTCAGGCTCGCGGCTTGGT
ThrSerAsnTyrAlaTyrThrLeuLysThrAlaAlaGlnAlaLysAspPheThrGlnLysAsnAlaGlyAlaGlnAlaAlaAlaLeuVal

1450    1460    1470    1480    1490    1500    1510    1520    1530
GTTTGGTAGTTGAATAGCTAGCTTTCTACTTGGCTGTCTATTATGTCAGGTGCCACTGTATTTATATCAAGGATATAAATACAGTGATGCC
PheGlySer***

1540    1550    1560    1570    1580    1590    1600    1610    1620
CTTTGTAATGTAAACAATTCTTCATGTTGAGATAAGCGGTGCCCATGTTAAACCTTGGGGTGCACTTGACAGATCCAATCTCTGTGGCTC

1630    1640    1650    1660    1670    1680    1690    1700    1710
TTTCGGTCAGTATCGAGTTGCTGCTGTCAGGTGCTATTTAATCTTCGCTTTTATTATTAAGCGTCAGCAAAAGGACATCATGAAACA

1720    1730    1740    1750    1760    1770    1780    1790    1800
TGCGAATCACTACCTCCACTCGCTGGACACGGCCGATGCGCTGAAAGTTAATGAGAATGACCTACGACGACACAGCGGTAGTGAGTCC

1810    1820    1830    1840    1850    1860    1870    1880    1890
TGACTTTCTGTGATGAGTGACACGGTCTTTATTTGGGACACCATGCCATTGCGTGAGCTGGATGGCAGCGGTGTATCTGTGGATGGCTG

1900    1910    1920    1930    1940    1950    1960    1970    1980
GTCAGTGATCTTACCTTGACGCGGACCGCTCGCCCCATGATCCACAATTTCATCAACGCGGACGGCGGTACGACATCAAGCGGATTG

1990    2000    2010    2020    2030    2040    2050    2060    2070
GGAAGATGCCATGGCCATGCGCGGATCTGCTACTGTTACTCGCGCACCGGCAAGACTGGATCTTGGCGCGCGGGTCTAGGCCCAAGG

2080    2090    2100    2110    2120    2130    2140    2150    2160
GGTTTCACCGACACGCGTGAGTGGGCGGCACGCGGATCTGCTCAATAACAACGGTGATATCGACCTGTATTACCTGCGTAACCCC

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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
<u>P. marginalis</u>	1- 60	MSYPESKLTGLTGFALAAKVTGGWAGPVVSIITNLDQLKANIGTVTPQVLVINSNISASSL
<u>E. carotovora</u>	1- 60	MAYPTTNLTGLIGFAAAKVTGGTGGKVVTNLSLADFKSAVSGSAKTIIVLGSLSKTSAL
<u>P. marginalis</u>	61-119	TKVNMGANKLTIGSF-QNRTLENIHLRATAQSQNIILQNLIFKHSANIKANDDIQVYLN
<u>E. carotovora</u>	61-120	TKVVFSGSKTIIVGSGFGANVLNIHLRAESNSSNVIFQNLVFKHDVAIKDNDIQLYLN
<u>P. marginalis</u>	120-179	GSKYWIHDCSFVGHWSITDGSSEKLLYIGEKADYATISNCFFGSHKYLIFGHPADDNN
<u>E. carotovora</u>	121-180	GKGYWVDHCSWPGHWSNDNGSLDKLIYIGEKADYITISNCLFSNHRYGCIIFGHPADDNN
<u>P. marginalis</u>	180-239	AAFNGYPRLTICHNRFDNMEVRAPGLMRYGYFHVYNNYINKFHLGFTLAQNA NILSESNY
<u>E. carotovora</u>	181-240	SAYNGYPRLTICHNYENIQVRAPGLMRYGYFHVFNYYNKFQLAFTVAQNA NVISERNV
<u>P. marginalis</u>	240-298	FGEGSQNNGLMDLKGSG-TFTDINSVPPIITNQKSPKAQWATSNNYAYLTKTAQAQKDF
<u>E. carotovora</u>	241-300	FGSGAEKKGWVDKNGSGTFTDNGSSPAAVASKSPAAKWTASSNYSYSLMTTAAQGSWV
<u>P. marginalis</u>	299-312	KNAGAQAALVFGS
<u>E. carotovora</u>	301-314	SNAGAQSALKFPS

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 controlled 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 DH5 α 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 DH5 α harboring pPNL6301. The expression of pnl gene in E.coli DH5 α 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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