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Molecular Characterization of an Inducible *p*-Coumaric Acid Decarboxylase from *Lactobacillus plantarum*: Gene Cloning, Transcriptional Analysis, Overexpression in *Escherichia coli*, Purification, and Characterization†

JEAN-FRANÇOIS CAVIN,* LISE BARTHELMEBS, AND CHARLES DIVIÈS
Laboratoire de Microbiologie, UA, INRA, ENSBANA, Université de Bourgogne, F-21000 Dijon, France

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By using degenerate primers designed from the first 19 N-terminal amino acids of *Lactobacillus plantarum* *p*-coumaric acid decarboxylase (PDC), a 56-bp fragment was amplified from *L. plantarum* in PCRs and used as a probe for screening an *L. plantarum* genomic bank. Of the 2,880 clones in the genomic bank, one was isolated by colony hybridization and contained a 519-bp open reading frame (*pdc* gene) followed by a putative terminator structure. The *pdc* gene is expressed on a monocistronic transcriptional unit, which is transcribed from promoter sequences homologous to *Lactococcus* promoter sequences. No mRNA from *pdc* and no PDC activity were detected in uninduced cell extracts, indicating that the expression is transcriptionally regulated by *p*-coumaric acid, which corresponds to an activation factor up to 6,000. The *pdc* gene was overexpressed constitutively in *Escherichia coli*, and the recombinant enzyme was purified and characterized.

Wines are generally produced by conducting two successive fermentations. The first one, alcoholic fermentation, is produced by yeasts selected for technical and aromatic qualities. The second one, malolactic fermentation, is the conversion of L-malic acid into L-lactic acid and CO_2 by lactic acid bacteria, especially *Leuconostoc oenos*. During wine making, aging, and storage, growth and metabolism of the indigenous flora of lactobacilli and pediococci may be responsible for changes in organoleptic properties and alteration of wine (9, 10).

Phenolic acids, also called cinnamic acid derivatives (principally *p*-coumaric and ferulic acids), are generally esterified with tartaric acid in grape must and wine and can be released as free acids during wine making by some cinnamoyl esterase activities (15). Most often, free phenolic acids are metabolized by different microorganisms into 4-vinyl derivates and then reduced into 4-ethyl derivates; alternatively, these phenolic acids can be reduced to substituted phenyl propionic acids and then decarboxylated to generate 4-ethyl derivatives (see metabolic pathways and reference in Fig. 1). These volatile phenols are potential contributors to the aroma of wine and other fermented foods and beverages. Sensory studies have shown that the ideal concentration in wine is about 2 mg/liter and that 4 mg/liter may negatively affect the perception of wine aroma (16). Among the different wine lactic acid bacteria, we have found that the ubiquitous lactic acid bacterium *Lactobacillus plantarum*, used as a malolactic starter (24, 25) in wine and as a lactic starter for many vegetable fermentations, was able to rapidly decarboxylate these acids into the corresponding 4-vinyl derivatives (4). We have shown that two inducible phenolic acid decarboxylases were present in this bacterium, and one of them, called *p*-coumaric acid decarboxylase (PDC), able to metabolize only *p*-coumaric and caffeic acid, was purified and characterized (5). It was found to be different in structure, specificity of substrate, expression, and N-terminal amino acid sequence from the two other recently purified and characterized bacterial *p*-coumaric and ferulic acid decarboxylases which were isolated from *Bacillus pumilus* (11) and from *Pseudomonas fluorescens* (21). PDC was also different from the phenylacrylic decarboxylase cloned from *Saccharomyces cerevisiae* (8).

In this paper, we describe cloning by using PCR amplification and involving data from N-terminal amino acid sequence (5) and transcriptional analysis of the gene that encodes a PDC in *L. plantarum*. We present the purification and the characterization of the overexpressed and stable recombinant enzyme in *Escherichia coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are described in Table 1. The homofermentative malolactic bacte-

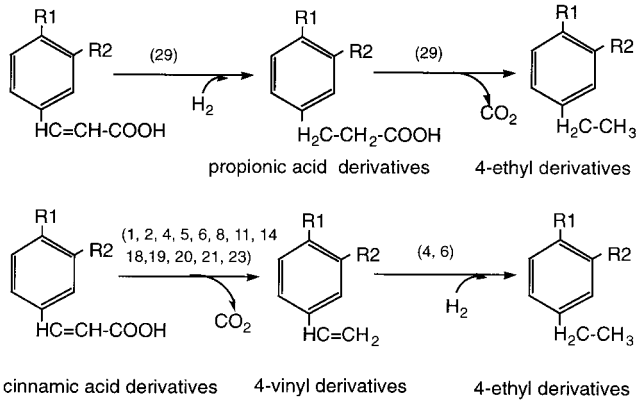

FIG. 1. Pathways for the biotransformation of cinnamic acid derivatives by microorganisms (numbers above arrows indicate references).

* Corresponding author. Mailing address: Laboratoire de Microbiologie, UA, INRA, ENSBANA, 1 esplanade Erasme, F-21000 Dijon, France. Phone: (33) 03.80.39.66.72. Fax: (33) 03.80.39.66.40. E-mail: cavinjf@u-bourgogne.fr.

† This paper is dedicated to the memory of Pierre Dubois, INRA Dijon, France.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli TG1	*supE hsd*Δ*5thi* Δ(*lac-proAB*)F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	17
L. plantarum LPCHL2	Wild type, gram positive, ubiquitous homolactic acid bacterium	Ch. Hansen Lab, Denmark (25)
Plasmids		
pJDC9	Em^r Δ*lacZ*	7
pTZ19R	Ap^r Δ*lacZ*	26
pTZN-PDC	pTZ19R containing the 56-bp PCR product corresponding to the N-terminal amino acid sequence of PDC, into the *Sma*I site	This study
pJPDC1	pJDC9 containing the 2.3-kb *Sau*3A fragment from *L. plantarum*	This study
pJPDC2	pJDC9 containing the 1.5-kb *Eco*RI-*Pst*I fragment of pJPDC1	This study
pJPDC3	pJDC9 containing the 1.0-kb *Eco*RI-*Hin*dIII fragment of pJPDC1	This study
pTZPDC1	pTZ19R containing the 2.3-kb *Sac*I-*Pst*I fragment of pJPDC1	This study

rium *L. plantarum*, maintained in MRS medium (12) with 30% (wt/vol) glycerol at −70°C, was grown statically in MRS medium, and cells were harvested for decarboxylating activity or total DNA extraction at A_{600} = 1 (400 mg/liter of dry biomass). *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth medium (3) or agar medium at 37°C with the appropriate antibiotics ampicillin or erythromycin (200 mg/liter).

DNA manipulation, sequencing, and computer analysis. Standard molecular procedures described by Sambrook et al. (27) were used. Double-stranded DNA from recombinant plasmids was purified by using the Qiagen plasmid kit (Tip 100, Diagen) and sequenced by the dideoxy chain termination method (28) with the T7 DNA polymerase sequencing kit (Pharmacia LKB Biotech). Both strands were sequenced by using specific synthetic primers (Eurogentec). Computer analyses of the sequences were carried out by using PC GENE (Intelligenetics) software.

Synthesis of a specific probe for the *L. plantarum* PDC gene by PCR. A PCR was performed in an automated Hybaid DNA thermocycler in two steps to obtain both a sufficient quantity of specific PCR products. For the first step, the reaction contained 10 μl of 10× *Taq* DNA polymerase buffer; 20 μM each dATP, dCTP, dGTP, and dTTP; 20 ng of genomic template DNA from *L. plantarum*; the two degenerate primers LPD1 (30 pM, 2,048 combinations) and LPD2 (10 pM, 768 combinations) (Fig. 2) deduced from the N-terminal sequence of the decarboxylase (5); and 0.5 U of *Taq* DNA polymerase (Appligene) in a final volume of 100 μl. DNA amplification was performed for 35 cycles consisting of denaturation for 1 min at 92°C, annealing for 1 min at 50°C, and elongation for 30 s at 72°C. After this first step, only a very small quantity of PCR product was obtained (<5 ng) and 2 μl of this product was used as a template DNA for the second step of PCR amplification under the same conditions. The PCR products were fractionated on a 1.5% (wt/vol) agarose gel electrophoresis with a DNA ladder as a reference (100 to 1,500 bp; Gibco BRL) and the expected band of 56 bp was recovered by excising and extraction from agarose, using the Jet-Sorb kit (Genomed, Bioprobe France). The DNA was blunt-ended by five cycles of PCR amplification with the *Pfu* (Stratagene) DNA polymerase before cloning into the *Sma*I site of the pTZ19R vector. The product of the amplification was sequenced with the reverse and universal primers to check its identity with the N-terminal sequence of the decarboxylase.

Southern blot and DNA hybridization. The PCR product extracted from agarose was radiolabeled with [α-^{32}P]dATP (Isotopchim, France) by 10 cycles of PCR amplification, as described above, by using the primer LPD4 (Fig. 2) deduced from the sequence of the PCR product to generate a highly radiolabeled single-stranded 48-base DNA. This probe was used for Southern blot hybridization with *L. plantarum* total DNA and for screening a *L. plantarum Sau*3A library.

Preparation and screening of the *L. plantarum* genomic library. Total DNA from *L. plantarum* was partially digested with restriction enzyme *Sau*3A and was size fractionated with a 10 to 40% (wt/vol) sucrose gradient by the methods described by Sambrook et al. (27). Fractions containing DNA fragments from 5 to 8 kb were used to build the library. DNA fragments were ligated to *Bam*HI-digested pJDC9 treated with bacterial alkaline phosphatase (Gibco BRL). Recombinant plasmids were transferred into *E. coli* TG1 cells by electroporation, and up to 2,900 recombinant clones were stored at −70°C in microtitration plates. Colony hybridization was carried out by transferring the recombinant clones from master plates to nylon membranes (Nytran; Schleicher and Schuell) on agar plates. The colonies on the filters were then lysed, and the liberated DNA was fixed to the filter by baking as previously described (22). The [α-^{32}P]dATP-labeled single-stranded DNA probe of 48 bases was used to screen the filters. Clones containing the N-terminal decarboxylase-coding DNA fragment were detected by exposure of the filters to X-ray film.

Isolation of total RNA from *L. plantarum*. Preparation of total RNA from *L. plantarum* was done with Tri-Reagent (Sigma). Cells were grown in MRS liquid medium until they reached 0.7 A_{600} units (280 mg/liter of dry biomass), and the culture was divided in two: the noninduced culture and the induced culture to which was added 200 mg of *p*-coumaric acid per liter. These two cultures were incubated for 40 min at 30°C. During this period, samples of 40 ml of cultures at 2, 5, 10, 20, and 40 min were refrigerated immediately in ice water and cells were harvested by centrifugation (8,000 × *g*, 10 min, 4°C). Cells were resuspended in 1 ml of ice-cold Tri-Reagent and put in a 2-ml screw-cap microtube containing 150 mg of glass microbeads (50 to 100 μm) to disrupt cells by 5 cycles of 1-min treatment in a mini bead-beater (Polylabo). After centrifugation, 0.7 ml of supernatant was twice extracted with chloroform and precipitated by the addition of 1 vol of isopropanol at room temperature for 10 min and washed twice in ethanol (70% [vol/vol]). The RNA pellet was dried under vacuum and resuspended in 30 to 50 μl of TE. The RNA concentration was determined by UV scanning (from 230 to 320 nm, maximal absorption peak at 260 nm), and its integrity was checked by standard denaturing agarose gel electrophoresis.

Northern blot analysis. Total RNA from *L. plantarum* was separated in denaturing formaldehyde agarose gels and transferred to nylon membranes by

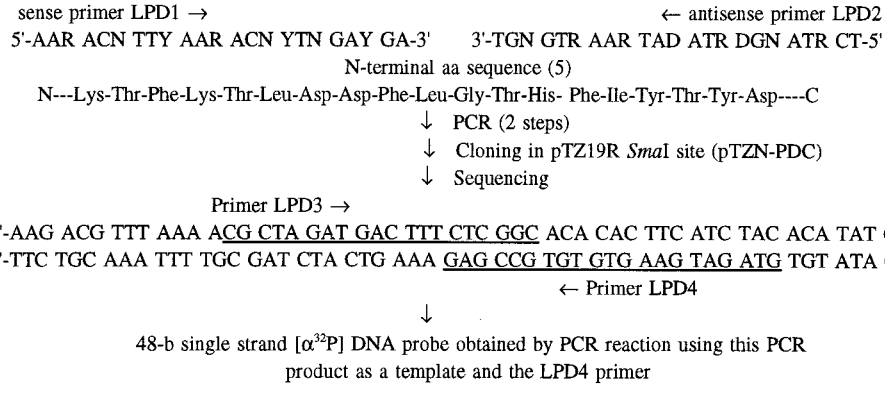

FIG. 2. Strategy to generate a specific probe for *p*-coumaric acid decarboxylase gene from *L. plantarum*.

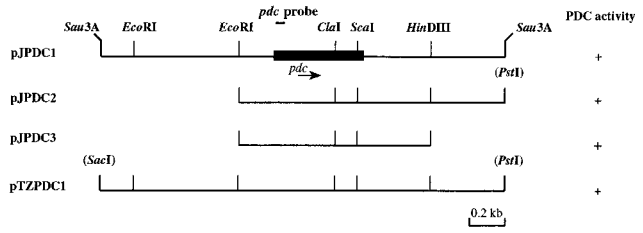

FIG. 3. Restriction enzyme map of the 2.3-kb insertion of recombinant plasmid pJPDC1. The subclones obtained with *Eco*RI, *Hin*dIII, *Pst*I, and *Sac*I are shown. On the right, the presence of *p*-coumaric acid decarboxylase activity (PDC) of the corresponding transformants is indicated. Restriction sites in brackets belong to the multicloning site of the vector.

using the Pharmacia Vacuum System as recommended by the manufacturer. Hybridization with radiolabeled PCR DNA *pdc* probes was performed as described for Southern hybridization. Size determination of the transcripts was done by using an RNA ladder (0.24 to 9.5 kb; Gibco BRL) as the standard.

Primer extension analysis. Primer extension analysis was done with two antisense primers (LPD4 and LPD16; see Fig. 4) located in the 5′ region of the *pdc* gene. Then, 4 μl of RNA (20 μg) was mixed with 20 pmol of primer (1 μl) and 20 μl of extension mix: 5 μl of 5× reverse transcriptase buffer; 5 μl of 0.1 M dithiothreitol; 0.25 μl each dCTP, dGTP, and dTTP (each at 100 mM); 0.25 μl of [α-^{32}P]dATP (Isotopchim); 1 μl of RNase inhibitor (40 U/μl, Boehringer Mannheim); and 13 μl of distilled water. Denaturation and annealing were performed by incubating the mix at 80°C for 5 min and at 50°C for 10 min. Then, the mix was placed at 37°C and the reverse transcriptase reaction was immediately started by addition of 10 U of MLV reverse transcriptase (Appligene) and incubation for 15 min. Then, 0.5 μl of dATP (100 mM) was added to the reaction and the incubation was continued for 45 min to ensure the complete synthesis of cDNA. Next, 3 μl of loading denaturing buffer (provided in T7 sequencing kit; Pharmacia) was added to 3 μl of the reaction mixture. The mixture was denatured at 80°C for 3 min and loaded onto a sequence gel with sequence reactions using the respective DNA with the same primers.

Preparation of cell extracts and enzyme assays. Cells of *L. plantarum* grown in MRS medium and of *E. coli* grown in LB medium were disrupted with a French press at 1.2×10^8 Pa, and decarboxylase activity was assayed by monitoring the disappearance of absorption peaks of the different substrates as previously described (5). The total protein concentration was determined with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard, and the specific activity was expressed as micromoles of substrate degraded per minute per milligram of protein.

PAGE analysis. The protein extracts containing PDC activity were resolved by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (14% resolving gel) as previously described (5) with molecular size markers (10- to 200-kDa protein ladder, Gibco-BRL) as standards.

Recombinant protein purification from *E. coli*. The enzyme purification was performed as previously described (5). Crude cell extract from concentrated and washed cells of *E. coli* TG1 (pJPDC1) grown in 1.5 liter of LB medium was obtained with the French press and then was fractionated by the addition of $(NH_4)_2SO_4$ [25, 35, and 45% (wt/vol) and saturation at 0°C]. The 45% fraction containing the highest specific activity was used for purification as previously described (5). As the PDC enzyme was overpressed in *E. coli*, only five steps of the seven-step procedure used for the purification of PDC from *L. plantarum* (5) were necessary for purification to SDS-PAGE homogeneity.

Nucleotide sequence accession number. The sequence of the DNA fragment containing the *pdc* gene has been deposited in the GenBank database under accession number U63827.

RESULTS AND DISCUSSION

Cloning of the PDC gene from *L. plantarum*. *L. plantarum* LPCHL2 PDC was purified by two-dimensional acrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane to determine the N-terminal amino acid sequence (5). This sequence was used to design two degenerate primers (LPD1 and LPD2) that were used in PCRs to amplify a 56-bp DNA fragment (N-PDC) (Fig. 2). This strategy was preferred to direct labeling of these very degenerate primers to obtain a specific probe for screening the genomic bank. The putative N-PDC fragment was cloned into the *Sma*I site of pTZ19R and sequenced. Its deduced amino acid sequence proved to be identical to the 19 amino acids of the PDC enzyme. This nucleotide sequence was used to synthesize new primers (LPD3 and LPD4) for sequencing and to construct a labeled single-stranded DNA probe as described above. The N-PDC probe hybridized with DNA from *L. plantarum* LPCHL2 and did not hybridize with DNA from *E. coli* TG1 (data not shown) and was used to screen the genomic library. Only one clone (pJPDC1) of the genomic library with a 2.3-kb insertion hybridized with the probe. This clone was able to decarboxylate *p*-coumaric acid at a high rate. This insertion containing the entire gene encoding the PDC activity was subcloned in pJDC9 and pTZ19R vectors for expression studies and sequencing (Fig. 3).

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GTTTTAAAATAAATTCATCTTTCGTAGCGGTTAAGTCTGGTGTACCGACACTGATCCACTCATGTAACAGCTGCCGACCAGTTGCGGTGATGTGGTAGAG   100

TTTCTTTTCAAGTTTTTCGCCACTGACCGTAATTTCATGCGTGACGTAACCTTGTTCTTCACTCGCTTCAATTCGGATAAATTTGACTATGTTGAGCTTG   200

CCAGAATTCACCGATCTCGTCATCAAAGGCTTTAGTTAGATCATAGCCCGTTAATGGTTGCTGGTTTAATAACCCTAGAATAATAAATGTAACTTGTTTT   300

                                  -35                 -10
TTTGCGCCATTGGAAATTCCTCCTTGACGATTCCTTGATAACAGTATACACTAACCTTGTTGTTTAAAACATGTAATAAATTACATGATTAAAACGACAT   400

                                                                LPD4                LPD16
ATTAGACATAAGGAAGGTAATTCTAATGACAAAAACTTTTAAAACACTTGATGACTTTCTCGGCACACACTTTATCTACACTTATGATAACGGCTGGGAA   500
          RBS    pdc →  M  T  K  T  F  K  T  L  D  D  F  L  G  T  H  F  I  Y  T  Y  D  N  G  W  E

TACGAGTGGTACGCCAAGAACGACCACACCGTTGATTACCGAATCCACGGTGGGATGGTTGCCGGTCGTTGGGTCACTGATCAAAAAGCTGACATCGTCA   600
 Y  E  W  Y  A  K  N  D  H  T  V  D  Y  R  I  H  G  G  M  V  A  G  R  W  V  T  D  Q  K  A  D  I  V

TGTTGACCGAAGGCATTTACAAAATTTCTTGGACTGAACCAACTGGGACTGACGTTGCACTAGACTTCATGCCCAATGAGAAGAAACTACACGGTACGAT   700
M  L  T  E  G  I  Y  K  I  S  W  T  E  P  T  G  T  D  V  A  L  D  F  M  P  N  E  K  K  L  H  G  T  I

TTTCTTCCCAAAGTGGGTTGAAGAACACCCTGAAATTACGGTCACTTACCAAAACGAACACATCGATTTAATGGAACAGTCTCGTGAAAAGTATGCCACT   800
  F  F  P  K  W  V  E  E  H  P  E  I  T  V  T  Y  Q  N  E  H  I  D  L  M  E  Q  S  R  E  K  Y  A  T

TATCCAAAACTAGTTGTACCCGAATTTGCCAATATTACTTACATGGGCGAGGGCCAAAACAATGAAGATGTAATCAGTGAAGCACCTTACAAAGAAATGC   900
 Y  P  K  L  V  V  P  E  F  A  N  I  T  Y  M  G  E  G  Q  N  N  E  D  V  I  S  E  A  P  Y  K  E  M

CGAATGATATTCGCAACGGCAAGTACTTGATCAAAACTACCATCGTTTAAATAAGTAATGTTTCACGTGAAACATTCTGGTCTACAAATTGGATAATTAG  1000
P  N  D  I  R  N  G  K  Y  L  I  K  T  T  I  V

CAGTCTGTCCAATCTTTGAAAATTACTAATCATCTGAAAAACCATTCATTTAGCAACACTTCAAAAAGAGGCCTTCATAAATGTCATGACATTTATGAAG  1100

GCTTTTTCGTGATTATTTTACTTAGGCTTCTGTTACATCCAGCGCAACCTGTCCCAGCCCGGCATCGAAGTCGGCTTGCCAGTGCCCCGCGGTCAAATGT  1200
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FIG. 4. Nucleotide sequence of the *pdc* gene. Numbers on the right refer to the nucleotide positions. The predicted amino acid sequence for the *pdc* gene is given below the DNA sequence. A putative ribosome-binding site (RBS) region is underlined. Initiation and stop codons are enclosed in boxes. The direction of transcription of the *pdc* gene (arrow) and the termination stem-loop structure (convergent arrows) are indicated. The transcription starting point is underlined. The deduced *pdc* gene promoter is indicated by solid bars below the −10 and −35 regions, joined by a thin line. Complementary sequences of the primers LPD4 and LPD16 used for primer extensions are indicated by arrows.

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PDCLP   MTKTFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQKA 55
                * *.* * ****.****** * *****.***** *** **** **
FDCBP           MDQFVGLHMIYTYENGWEYEIYIKNDHTIDYRIHSGMVGGRWVRDQEV 48

PDCLP   DIVMLTEGIYKISWTEPTGTDVALDFMPNEKKLHGTIFFPKWVEEHPEITVTYQN 110
         ** ** *.**.********** * *** **.** ******* * *.*** ***
FDCBP   NIVKLTKGVYKVSWTEPTGTDVSLNFMPEEKRMHGVIFFPKWVHERPDITVCYQN 103

PDCLP   EHIGLMEQSREKYATYPKLVVPEFANITY-MGEGQNNEDVISEAPYKEMPNDIRN 164
        . * **  ***** **** ****** ***    * * * .*.****  *  .**
FDCBP   DYIDLMKESREKYETYPKYVVPEFADITYIHHAGVNDETIIAEAPYEGMTDEIRA 158

PDCLP   GKYLIKTTIV 174
        *
FDCBP   GRK         161
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FIG. 5. Comparison of the deduced amino acid sequence of the *pdc* gene of *L. plantarum* (PDCLP) with the sequence of the FDC *B. pumilus* protein (FDCBP; protein sequence accession number X84815). The sequences were aligned by using the Palign program. *, identity; ·, similarity. The numbers on the right are the amino acid positions in the protein sequences.

Nucleotide and protein sequences. The 2.3-kb insertion in recombinant plasmid pJPDC1 was sequenced on both strands. An open reading frame (ORF) of 625 bp was detected (Fig. 4) in the middle of the insertion. The coding region of this ORF was 522 bp long with a 5′ region corresponding to the N-terminal sequence of the PDC enzyme, and only a few single-base mismatches occurred in the PCR product in the 5′ region of the two degenerate primers LPD1 and LPD2. Upstream and downstream of this ORF corresponding to the *pdc* gene, two putative ORFs of ca. 500 bp were observed (data not shown) but no homology was found by multiple alignment in the EMBL Bank. A comparison of the primary structure of the deduced PDC protein sequence (174 amino acids) revealed an identity of 67% with the ferulic acid decarboxylase of *B. pumilus* (30) (Fig. 5). The second best score of the protein alignment was less than 25% identity. No homology was found with other known decarboxylases, including the phenylacrylic acid (cinnamic acid) decarboxylase (PAD1) cloned from *Saccharomyces cerevisiae*, which was able to decarboxylate, at a very low rate, ferulic and *p*-coumaric acids (8). A putative Shine-Dalgarno sequence was observed 12 bp upstream of the initiation codon ATG. Its sequence, 5′-AAGGAAGGT-3′, is complementary to the 3′ extremity of the 16S rRNA from *L. plantarum* (EMBL accession no. M58827). Further upstream of this ORF, potential −35 (TTGACG) and −10 (TACACT) promoter sequences were found (positions 324 to 329 and 347 to 352, respectively) that were identical to promoter sequences in *Lactococcus* (13). At 120 nucleotides beyond the TAA stop codon, a stable stem-loop structure (position 1071 to 1102) with an estimated ΔG of −28.4 kcal/mol that is followed by a stretch of T residues may function as a rho-independent terminator. The sequence alignment of PDC from *L. plantarum* with FDC of *B. pumilus* shows that the main differences between the two proteins are located in the N- and C-terminal parts. Sequencing the N-terminal amino acid region of the PDC protein purified from *L. plantarum* (5) clearly gave a lysine residue as the first amino acid of the protein (Fig. 2), which indicated that Met-1 and Thr-2 were missing from the purified enzyme. However, it is not known whether this was a result of nonspecific proteolysis or whether it was a posttranslational processing step.

Transcriptional analysis. Identical results were obtained from primer extension experiments performed with the two primers LPD4 and LPD16, using RNA from induced cultures of *L. plantarum* in MRS medium (with addition to the culture of *p*-coumaric acid 5 min before harvesting cells) as a template. They permitted identification of a G as a transcription starting point 64 nucleotides upstream of the putative initiation codon ATG (Fig. 6) and reinforced the hypothesis about the promoter sequences (Fig. 4). No primer extension product was detected by using RNA from the uninduced culture. Northern blot hybridization using the same templates was performed to

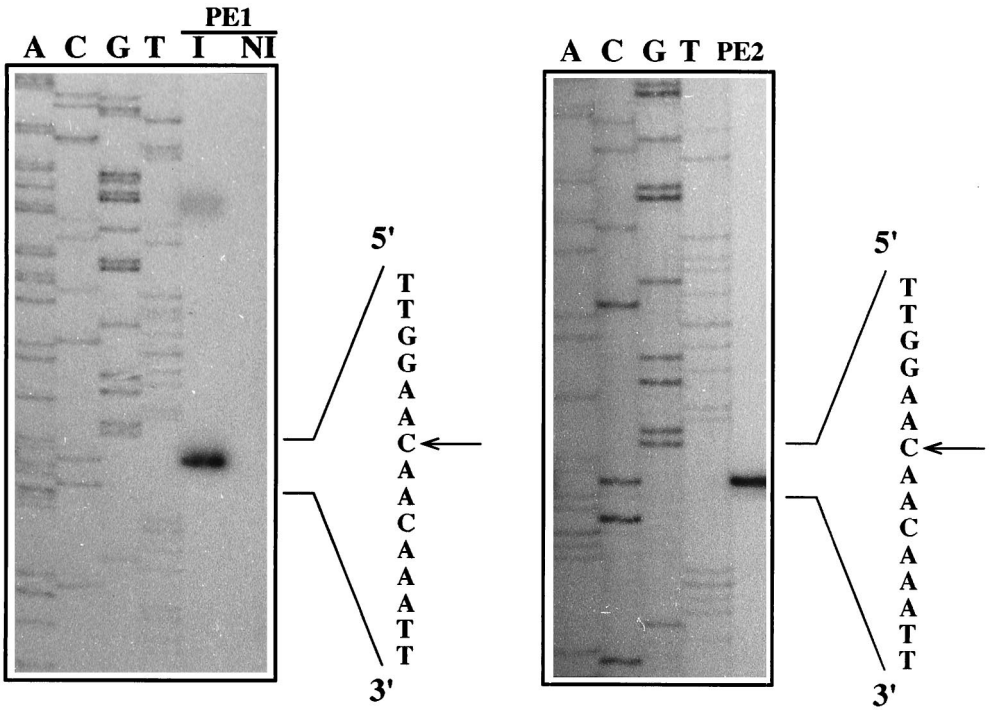
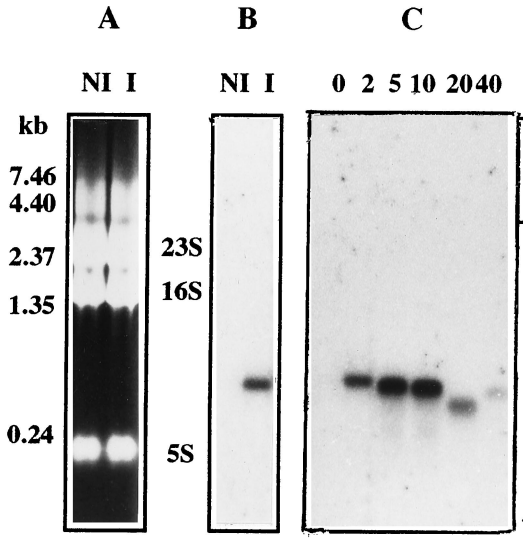


FIG. 6. Mapping of the 5′ end of *pdc* mRNA by primer extension analysis using primer LPD4 (PE1) with total RNA from uninduced (NI) and induced (I) cells (addition of 200 mg of *p*-coumaric acid per liter) from *L. plantarum* and primer LPD16 (PE2) with RNA from induced cells. The products of the reverse transcriptase reactions were analyzed by 6% (wt/vol) sequencing gel reactions of ACGT with the same primers. Arrow indicates the 5′ end of *pdc* gene mRNA.



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Sampling time (min)	*p*-coumaric acid (mg/l)	Specific activity ($\mu mol.min^{-1}.mg^{-1}$)
0	200	$<10^{-4}$
2	160	0.13
5	50	0.37
10	0	0.50
20	0	0.46
40	0	0.38

FIG. 7. (A) Denaturing agarose gel electrophoresis of total RNA (10 μg per lane) from *L. plantarum* uninduced (NI) and induced (I) cells (addition of *p*-coumaric acid 10 min before harvesting the cells). (B) Corresponding Northern blot analysis. (C) Northern blot analysis of total RNA purified from cells harvested after 0, 2, 5, 10, 20, and 40 min after the addition of 200 mg of *p*-coumaric acid per liter. Northern blot analysis was performed by using a single-strand DNA fragment complementary to the mRNA *pdc* gene as a probe. This probe was simultaneously obtained and labeled by PCR with pJPDC1 preparation as a template. (D) Residual *p*-coumaric acid concentration and specific PDC activity of each sample.

determine the size of the corresponding mRNA (Fig. 7A). No transcript and no PDC activity ($<10^{-4}$ U · mg^{-1}) were detected in the lane corresponding to the RNA extract and in the protein extract from uninduced cells, respectively. A single transcript of approximately 700 nucleotides, a size corresponding to a DNA fragment from the starting site point to the stem-loop of the *pdc* gene, was detected in the RNA extract from induced cells. The induced cell extract exhibited a high specific PDC activity of 0.5 μmol · min^{-1} · mg^{-1}. These results indicate that the *pdc* gene corresponds to a monocistronic structure independent of the putative ORFs upstream and downstream of the *pdc* gene. This also demonstrates that there is a transcriptional regulation of the *pdc* gene. The level of mRNA at different sampling times (0, 2, 5, 10, 20, and 40 min) after adding *p*-coumaric acid to the culture was studied by Northern hybridization as described above (Fig. 7B). The pool of *pdc* mRNA was maximal after 10 min of incubation and decreased rapidly (20- and 40-min samples) after *p*-coumaric acid was entirely metabolized (10 min) (the sample corresponding to 20 min presents a little artifact of gel migration). Maximal PDC activity was observed after 10 min of induction, after which the activity decreased slowly, corresponding to simple dilution of the enzyme in the growing cells during the last 40 min of the experiment.

Expression in *E. coli* and purification of the recombinant PDC. The different *E. coli* TG1 subclones (Fig. 3) expressed PDC activity at nearly the same level, independently of the *p*-coumaric acid and IPTG inducers (data not shown). No PDC activity was found in the control *E. coli* TG1 (pJDC9). The PDC protein was purified from *E. coli* TG1 (pJPDC1) recombinant culture. Crude extracts from the control *E. coli* TG1 (pJDC9), from the uninduced and induced (addition of *p*-

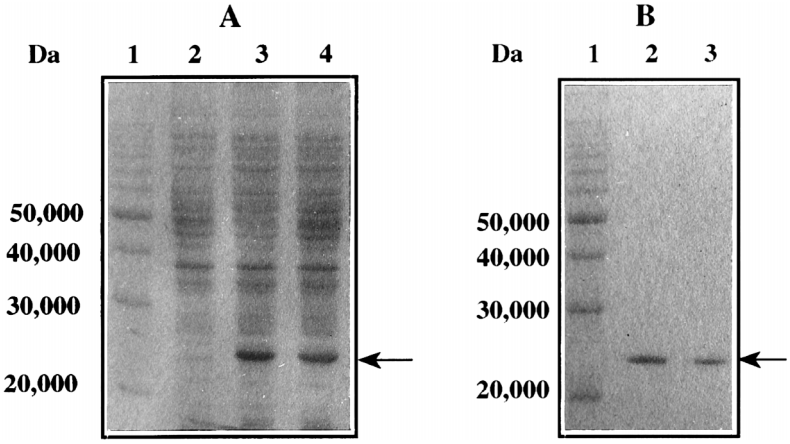

FIG. 8. (A) SDS-PAGE of crude cell extracts from *E. coli*. Lanes: 1, molecular mass standards (10-kDa protein ladder, Gibco BRL); 2, crude extract from *E. coli* TG1 (pJDC9); 3, crude extract from uninduced culture of *E. coli* TG1 (pJPDC1); 4, crude extract from induced (addition of 200 mg of *p*-coumaric acid per liter to the culture) *E. coli* TG1 (pJPDC1). (B) SDS-PAGE of the PDC purified from *E. coli* and from *L. plantarum*. Lanes: 1, molecular mass standards; 2, peak fraction from the Q Sepharose (pH 7.2) column from *E. coli* (2 μg); 3, peak from the Sephacryl HR200 fraction from *L. plantarum* (1.5 μg) (5).

coumaric acid) *E. coli* TG1 (pJPDC1) cultures were obtained with the French press and were subjected to SDS-PAGE (Fig. 8A). Similar profiles were obtained, except for the presence of an intense band in the recombinant (uninduced and induced culture) *E. coli* carrying the *pdc* gene. The presence of this band with an apparent molecular mass of 23.5 kDa, which was absent in the control, was correlated with the presence of a high PDC specific activity (8.5 μmol min^{-1} mg^{-1}). This activity was about 17-fold higher than the maximal PDC activity in the *L. plantarum*-induced cell extract, and the recombinant PDC was purified as previously described (5) to SDS-PAGE apparent homogeneity (Fig. 8B). At the end of the purification (data not shown), about 300 μg of 93-fold purified PDC with a specific activity of 792 μmol min^{-1} mg^{-1} was obtained.

Characterization of the recombinant PDC. By size exclusion chromatography (data not shown), the recombinant PDC had a molecular mass of 93 kDa, indicating that it was a homotetramer consisting of four 23.5-kDa subunits. The recombinant PDC displayed the same biochemical and enzymatic characteristics (K_m, V_m, substrate specificity and stability) as those of the enzyme purified from *L. plantarum* (5).

The structural and functional differences between the PDC enzyme from *L. plantarum* and the ferulic acid decarboxylase from *Bacillus pumilus* could be linked to the major differences in the N- and C-terminal sequences of the proteins. Mutagenesis of the *pdc* gene is currently being studied to correlate the amino acid sequence to the structure, substrate specificity, and metabolic characteristics of the recombinant proteins. If conserved protein sequences will be found in these decarboxylases, they could be used to determine catabolic sites and to synthesize probes in order to detect microorganisms in wine and food by PCR amplifications. It could be possible to obtain genetically modified strains: decarboxylase-overproducing and constitutive strains for enzyme and aroma production in biotechnological applications or disrupted strains as starters for wine and other fermented foods. The absence of detectable PDC activity correlated with the absence of detectable corresponding mRNA in *L. plantarum* uninduced cells, making this regulatory system a potential tool for gene expression studies in lactic acid bacteria or other gram-positive bacteria.

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