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Inducible Metabolism of Phenolic Acids in *Pediococcus pentosaceus* Is Encoded by an Autoregulated Operon Which Involves a New Class of Negative Transcriptional Regulator

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Pediococcus pentosaceus **displays a substrate-inducible phenolic acid decarboxylase (PAD) activity on *p*-coumaric acid. Based on DNA sequence homologies between the three PADs previously cloned, a DNA probe of the *Lactobacillus plantarum pdc* gene was used to screen a *P. pentosaceus* genomic library in order to clone the corresponding gene of this bacteria. One clone detected with this probe displayed a low PAD activity. Subcloning of this plasmid insertion allowed us to determine the part of the insert which contains a 534-bp open reading frame (ORF) coding for a 178-amino-acid protein presenting 81.5% of identity with *L. plantarum* PDC enzyme. This ORF was identified as the *padA* gene. A second ORF was located just downstream of the *padA* gene and displayed 37% identity with the product of the *Bacillus subtilis yfiO* gene. Subcloning, transcriptional analysis, and expression studies with *Escherichia coli* of these two genes under the *padA* gene promoter, demonstrated that the genes are organized in an autoregulated bicistronic operonic structure and that the gene located upstream of the *padA* gene encodes the transcriptional repressor of the *padA* gene. Transcription of this *pad* operon in *P. pentosaceus* is acid phenol dependent.**

Microorganisms generally respond to changes in environmental conditions through the actions of specific systems which detect physical or chemical changes and develop coordinated cellular responses to adapt to new conditions. Particularly, microorganisms can resist toxic compounds by various responses which are activated upon exposure to stress. Most of the time, detoxification involves either active efflux of the toxic compound from the cell by highly specific systems (3, 25) or enzymatic conversion of the toxic compound into a less toxic form (32). For some microorganisms, weak acids are considered to be the major natural toxic compounds. At low pH, they strongly inhibit growth by decreasing internal pH (29, 42). Phenolic acids, also called substituted hydroxycinnamic acids, are abundant in the plant kingdom because they are involved in the structure of plant cell walls (19) and are released by hemicellulases produced by several fungi and bacteria (13). Surprisingly, phenolic acids are not potentially toxic to all microorganisms. Some *Pseudomonas* strains (24, 33), as well as *Acinetobacter calcoaceticus* (38), are able to use them as the sole source of carbon for growth. They also serve as a signal and induce *vir* gene expression in the plant-associated *Agrobacterium tumefaciens* (27, 30). Nevertheless, they display antimicrobial activity against these three bacteria at a concentration above 0.5 mM (27), as well as acting against many other bacteria and fungi (5, 14, 42). Very little is known about the mechanisms evolved by microorganisms to counteract phenolic acid toxicity. Chambel et al. (11) showed that *Saccharomyces cerevisiae* induced the expression of the H^+-ATPase pumps in response to inhibitory concentrations of cinnamic acid. In a previous work (4), we demonstrated that the ubiquitous lactic acid bacterium *Lactobacillus plantarum* exhibits inducible *p*-coumaric acid decarboxylase (PDC) activity, which converts *p*-coumaric acid into 4-vinyl phenol, a less toxic compound. We also showed that *L. plantarum* PDC activity confers a selective advantage for growth in *p*-coumaric acid-supplemented medium and therefore proposed that PDC synthesis could be considered as a stress response induced by phenolic acid toxicity.

Several microorganisms such as *S. cerevisiae* (14), *Brettanomyces anomalus* (21), *L. plantarum*, and *Pediococcus pentosaceus* (7) have been reported to decarboxylate phenolic acids into 4-vinyl derivatives, which could then be reduced to 4-ethyl derivatives. These volatile phenols are valuable intermediates in the biotechnological production of new flavor and fragrance chemicals, but they are also regarded as sources of phenolic off-flavors in many beers and wines, due to their characteristic aroma and their low threshold detection (4). To date, only three bacterial phenolic acid decarboxylases (PADs) have been purified, characterized, and cloned: a ferulate decarboxylase (FDC) from *Bacillus pumilus* (41), a PDC from *L. plantarum* (8), and a PAD from *B. subtilis* (10). Although they exhibit 66% amino acid sequence identity, the purified enzymes have different structures, biochemical characteristics, and substrate specificities (10). They also differ from the phenylacrylic decarboxylase of *S. cerevisiae* (14). Unlike the fungal PADs of *S. cerevisiae* and *B. anomalus*, which are constitutively expressed at a low level (about 1 to 10 $nmol \cdot min^{-1} \cdot mg^{-1}$) (14, 21), the PADs of *L. plantarum* and *B. subtilis* have substrate-inducible decarboxylase activities of about 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ in the presence of their respective substrates. Transcriptional analyses showed that *pdc* and *pad* mRNA could not be detected in uninduced cell extracts, in agreement with the absence of PAD activity in the same extracts. Our results also indicated that expression of these two genes is transcriptionally activated up to 6,000-fold in the presence of phenolic acids (8, 10). These regulatory systems involving phenolic acids which are considered as natural compounds as opposed to classical chemical inducers, could constitute a useful tool for the study of gene expression in lactic acid bacteria and other gram-positive bacteria.

In order to improve our understanding of phenolic acid

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biodegradation, we have screened bacteria which encounter phenolic acids in their environment and which are able to metabolize these compounds. In the course of our screening, we found a strain of *P. pentosaceus*, a lactic acid bacterium isolated from wine, which was able to decarboxylate *p*-coumaric acid and could then be involved in aroma changes and alterations in vegetable fermented products. In this paper, we describe the cloning of the corresponding *padA* gene encoding a PAD, and we report the first cloning and characterization of a *pad* transcriptional regulator, named *padR*, which forms an autoregulated operonic structure with the *padA* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. pentosaceus* (strain PP1) was isolated in the laboratory from an aging Pinot noir red Burgundy wine and was identified with the API 50CHL system (BioMérieux, Marcy l'Etoile, France). The strain was grown in MRS medium (17) at 30°C without agitation. *Escherichia coli* strain TG1 was used as a host for construction of the genomic library and for subsequent cloning steps and was grown aerobically in Luria-Bertani (LB) medium (36) or agar medium at 37°C. Plasmid pTZ19R (35) was used as a vector for the library and for the subcloning steps. Plasmid pJDC9 (12) was used for subcloning steps. When appropriate, ampicillin or erythromycin (100 mg/liter) was added to the medium.

DNA manipulation, sequencing, and computer analysis. Standard molecular procedures described by Sambrook et al. (36) were used. Double-stranded DNA from recombinant plasmids was purified with the Qiagen plasmid kit (Tip 100; Qiagen, Hilden, Germany) and sequenced by the dideoxy chain termination method (37) with the ThermoSequenase cycle sequencing kit (Amersham, Life Science, Inc., Cleveland, Ohio). Both strands were sequenced by using specific synthetic primers (Gibco-BRL, Gaithersburg, Md.). Computer analyses of the sequences were carried out with PC/GENE software (Intelligenetics).

Preparation and screening of the *P. pentosaceus* genomic library with a *pdc*-specific probe from *L. plantarum*. Total DNA from *P. pentosaceus* was completely digested by *Hin*dIII, and the resulting DNA fragments were ligated to *Hin*dIII-digested pTZ19R treated with bacterial alkaline phosphatase (Gibco-BRL). The ligation mixture was transferred into *E. coli* TG1 cells by electroporation, and up to 1,500 recombinant clones containing plasmids with 1- to 7-kb DNA inserts were stored at −70°C in microtitration plates. In order to synthesize a *pdc*-specific probe, PCR was performed in an automated Hybaid DNA thermocycler by standard procedures with genomic DNA from *L. plantarum* as the template and the two oligonucleotides LPPDC3 (5′-CACTTGATGACTTTCT CGGCAC-3′) and LPPDC8 (5′-CTTCAACCCACTTTGGGAAG-3′) (8). The 300-bp PCR product was purified by agarose gel electrophoresis and extraction, by using the Jet-Sorb kit (Genomed, Bioprobe, Montreuil, France). The purified fragment was sequenced to confirm its identity and was radiolabeled with [α-^{32}P]dATP (NEM, Boston, Mass.) by random priming (Gibco-BRL kit). Colony hybridization was carried out at 60°C for 5 h, followed by 5 h at 50°C, using standard procedures as previously described (8). Clones that hybridized with the *pdc* probe were detected by exposure of the membranes to Kodak BIOMAX MS films.

Isolation of total RNA from *P. pentosaceus* and from recombinant *E. coli* strains. For *P. pentosaceus*, cells were grown in 600 ml of MRS medium to an optical density at 600 nm of 0.7, and the culture was divided in two: a noninduced subculture and an induced subculture to which 2.4 mM *p*-coumaric acid was added. These cultures were incubated for 120 min at 37°C. During this period, 100-ml samples were quickly removed and refrigerated in ice-water. Total RNAs were extracted and quantified as previously described (8). The RNA integrity was checked by standard denaturing agarose gel electrophoresis. For *E. coli* recombinant clones, cells were grown in 100 ml of LB medium with the appropriate antibiotic, and 30 ml of culture was treated as described above to obtain total RNA.

Northern blot and primer extension analysis. Total RNAs were separated in denaturing formaldehyde agarose gels and transferred to nylon membranes by using the Pharmacia vacuum system. Hybridization was performed at 60°C with an [α-^{32}P]dATP radiolabeled probe synthesized in a PCR mixture. Determination of the sizes of the transcripts was done by using an RNA ladder (0.24 to 9.5 kb; Gibco-BRL) as the standard. Primer extension analysis was done with two antisense primers, PPPAD5 and PPPAD11, located in the 5′ region of the *padA* gene. Reverse transcription was realized at 42°C with Superscript II reverse transcriptase (RT) (Gibco-BRL) as previously described (10). Three microliters of loading denaturing buffer was added to 3 μl of the reaction mixture. The mixture was denatured at 80°C for 3 min and loaded onto a 6% polyacrylamide gel in parallel with sequencing reactions with the *padA* DNA as the template and the same primers. For comparison of band intensity, autoradiography results were scanned and digitized. Band intensity was quantified with Bio1D software (Vilber Lourmat).

RT-PCR. To remove any contaminating DNA, 1 μg of total RNA was incubated at room temperature with 1 U of RNase-free DNase I (Gibco-BRL). Residual DNase I was inactivated at 65°C for 10 min. DNase I-treated RNA was subjected to reverse transcription into cDNA with Superscript II RT (Gibco-BRL) as recommended by the manufacturer. Ten percent of the total cDNA was then PCR amplified with *Taq* DNA polymerase (Appligene) by using the primers PPPAD4 and PP33, and 1/10 of the PCR products were run on a 1% (wt/vol) agarose gel stained with ethidium bromide (0.5 mg/liter). As a control, PCR of DNase-treated RNA was performed with the same primers to check for any DNA contamination.

Preparation of cell extracts, enzyme assays, and protein electrophoresis. Cells of *P. pentosaceus* and recombinant *E. coli* strains, grown in MRS and LB medium, respectively, were harvested and washed by centrifugation and then resuspended in phosphate buffer to test PAD activity. Cell extracts were obtained by disrupting concentrated cell suspensions (10 g [dry mass] per liter) with the French press at 1.2×10^8 Pa. PAD activity was assayed by monitoring the disappearance of absorption peaks of the different substrates and the simultaneous appearance of new peaks corresponding to vinyl derivatives as previously described (4, 7, 16). The total protein concentration was determined with a protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard, and the specific activity was expressed as micromoles or nanomoles of substrate degraded per minute per milligram of protein. The protein extracts containing PAD activity were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% resolving gel) as previously described (9) with molecular weight markers (SDS-PAGE standards, low range; Bio-Rad) as standards.

Nucleotide sequence accession number. The sequence of the 3,032-bp DNA fragment containing the *padA* and the *padR* genes has been deposited in the EMBL nucleotide sequence database under accession no. AJ276891.

RESULTS

Expression of PAD activity in *P. pentosaceus*. A preliminary experiment revealed that *P. pentosaceus* was able to decarboxylate *p*-coumaric acid used to supplement MRS medium. An approximately equimolar concentration of 4-vinyl phenol was found in the growth medium, indicating that this derivative did not accumulate in the cells (data not shown), as previously shown for *L. plantarum* (8). Resting cells and crude cell extracts were obtained from *P. pentosaceus* cultures, either induced with 1.2 mM *p*-coumaric acid or uninduced, and were tested for PAD activity on *p*-coumaric acid. No PAD activity was detected in the uninduced cells and corresponding cell extracts. Induced cells displayed decarboxylase activity on *p*-coumaric acid, whereas no detectable activity could be found in the corresponding cell extract. Since we previously showed that the *L. plantarum* PDC enzyme required ammonium sulfate or NaCl to decarboxylate ferulic acid in vitro (4), 200 g of ammonium sulfate per liter was added to induced cell extracts of *P. pentosaceus*, which proved able to restore PAD activity.

To determine whether PAD activity would confer phenolic acid resistance to *P. pentosaceus* cells, three concentrations of *p*-coumaric acid (1.2, 3, and 6 mM) were added to *P. pentosaceus* cells cultured in MRS broth at different initial pHs (6.5, 5.5, and 4.5). The results (data not shown) were similar to those observed with the *L. plantarum* LPNC8 wild-type strain grown under the same conditions (4). At pH 6.5, addition of 1.2 or 3 mM *p*-coumaric acid had no apparent effect on growth. Addition of 6 mM *p*-coumaric acid increased the latency period, but when all available *p*-coumaric acid was metabolized, the final biomass was the same as that of the culture without *p*-coumaric acid. An increase in the latency period was observed when the initial pH of growth decreased. These results indicate that *p*-coumaric acid is toxic for *P. pentosaceus*, particularly at a low pH value, and that the PAD activity induced in the latency period which metabolized this toxic substrate probably allows *P. pentosaceus* cells to thwart phenolic acid toxicity, as we have demonstrated with a *pdc*-deleted mutant strain of *L. plantarum* (4).

Cloning and sequencing of the PAD gene from *P. pentosaceus*. Based on the high degree of conservation among known PADs (8, 10, 41) and because *P. pentosaceus* is phylogenetically closed to *L. plantarum* (18), a rapid strategy was adopted to

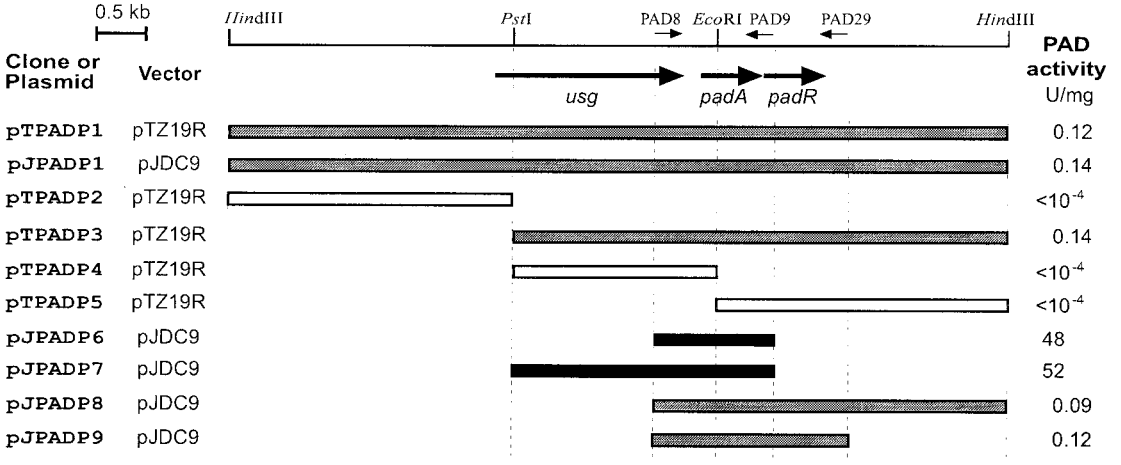

FIG. 1. Physical map of the *padAR* locus and delineation of subcloned fragments. The parent plasmid pTPADP1 was isolated from the *P. pentosaceus* genomic library. Restriction sites and primers (small horizontal arrows) used to obtain the different subclones are shown. The ORFs identified by sequencing are indicated by large arrows. PAD activity measured on *p*-coumaric acid in *E. coli* is indicated to the right of each subclone (U, micromoles of *p*-coumaric acid degraded per minute).

test whether a DNA probe from *L. plantarum pdc* could be used to screen a *P. pentosaceus* genomic library. A preliminary Southern hybridization experiment at 50°C showed that a DNA probe encompassing the first 300 bp of the *L. plantarum pdc* gene hybridized weakly but specifically with one band of about 6 kb from *P. pentosaceus* total DNA digested with *Hin*dIII (data not shown). The same probe was used to screen a *P. pentosaceus* genomic library in *E. coli*. One clone from the genomic library clearly hybridized with the *pdc* probe and was designated TG1(pTPADP1). Genetic and biochemical characterization indicated that pTPADP1 contained a 6-kb-long insert which conferred a PAD activity of 100 nmol · min^{-1} · mg^{-1} on *p*-coumaric acid. Various subfragments of the full-length insert were subcloned in pTZ19R to localize the *padA* gene by measuring the PAD activity of each subclone (Fig. 1). The results indicated that the PAD-encoding gene was likely interrupted by the *Eco*RI site located at the junction between TG1(pTPADP4) and TG1(pTPADP5) subclones (Fig. 1).

Sequencing was initiated from the *Eco*RI side of the two plasmids. An open reading frame (ORF) with a coding capacity of 528 bp was detected, preceded by a putative ribosome binding site (underlined) (5′-AAAGGGG-3′) complementary to the 3′ extremity of the 16S rRNA from *L. plantarum* (3′-U UUCCUCCA-5′) (EMBL accession no. M58827) and encoding a 178-amino-acid deduced product of approximately 25 kDa. No dyad symmetry region that could act as a transcriptional terminator was found downstream the TAA stop codon. A region of dyad symmetry was identified upstream from the start codon (Fig. 2). The putative ORF shares 81.5, 67, and 64% amino acid sequence identity with the PDC from *L. plantarum*, PAD from *B. subtilis*, and FDC from *B. pumilus*, respectively, with the less-conserved domains located in the N- and C-terminal regions (Fig. 3). It was therefore identified as the *padA* gene. Twenty-six nucleotides (nt) downstream from the *padA* stop codon, a second ORF, designated OrfX and transcribed in the same direction, was identified, with a coding capacity of 177 residues. For reasons that will become clear in the following sections, this second ORF was designated *padR*. It is preceded by a conventional ribosome binding site (5′-GGAGA-3′) and followed by a putative transcriptional terminator with a theoretical ΔG of −19.7 kcal/mol (41) (Fig. 2). Upstream from the *padA* gene, a 1,437-bp ORF designated *usg* and transcribed in the same orientation could encode a 479-amino-acid product which does not display any significant homology with any protein sequence available in the databases.

The *padA* gene belongs to an operon which is transcriptionally regulated by phenolic acids. Northern blot hybridization experiments were undertaken in order to study the transcriptional regulation of the *padA* gene. RNA samples from uninduced and *p*-coumaric-induced cultures were prepared to determine the size and level of the *padA* mRNA at different sampling times after addition of the inducer (Fig. 4A). No transcript was detected in the lane corresponding to the mRNA extracted from uninduced cells. A single transcript of approximately 1,200 nt was detected in the RNA extracted from cells induced with *p*-coumaric acid and was maximal after 10 min of induction. The level of *padA* transcript had significantly decreased after 40 min of incubation and was no longer detectable after 2 h. At these two later time points, the inducer was totally degraded. Primer extension experiments were performed with primers PPPAD5 (Fig. 4B) and PPPAD11 (data not shown), using RNA from *p*-coumaric-induced cells harvested after 10 min of induction. Identical results were obtained with both primers, allowing the identification of G residue, located 42 nt upstream from the start codon, as the transcription start site (Fig. 4B). No primer extension product was detected with RNA from the uninduced culture as the template (Fig. 4B). The size of the *padA* transcript of approximately 1,200 bp indicated that *padA* and *padR* appeared to be transcribed as a single transcription unit (Fig. 2). To confirm this hypothetical operonic organization, RT-PCR was carried out with mRNA prepared from *P. pentosaceus* grown with and without *p*-coumaric acid (2.4 mM) by using the primers PPPAD4 and PP33, located within the *padA* and *padR* genes, respectively (Fig. 2). A PCR product of the expected size (619 bp) (Fig. 5) and sequence (data not shown) was obtained with mRNA from an induced culture as the template, supporting the operonic arrangement of *padA* and *padR*. A very weak amplification product was detected with RNA from uninduced cells, confirming substrate-mediated regulation of the operon. No PCR products were detected in control reactions that were designed to detect chromosomal DNA contamination. To further confirm the operonic organization of the two genes, primer extension experiments were performed with total RNA from uninduced and induced *P. pentosaceus* cells with antisense primer PP91 internal to *padR* (Fig. 2). No primer exten-

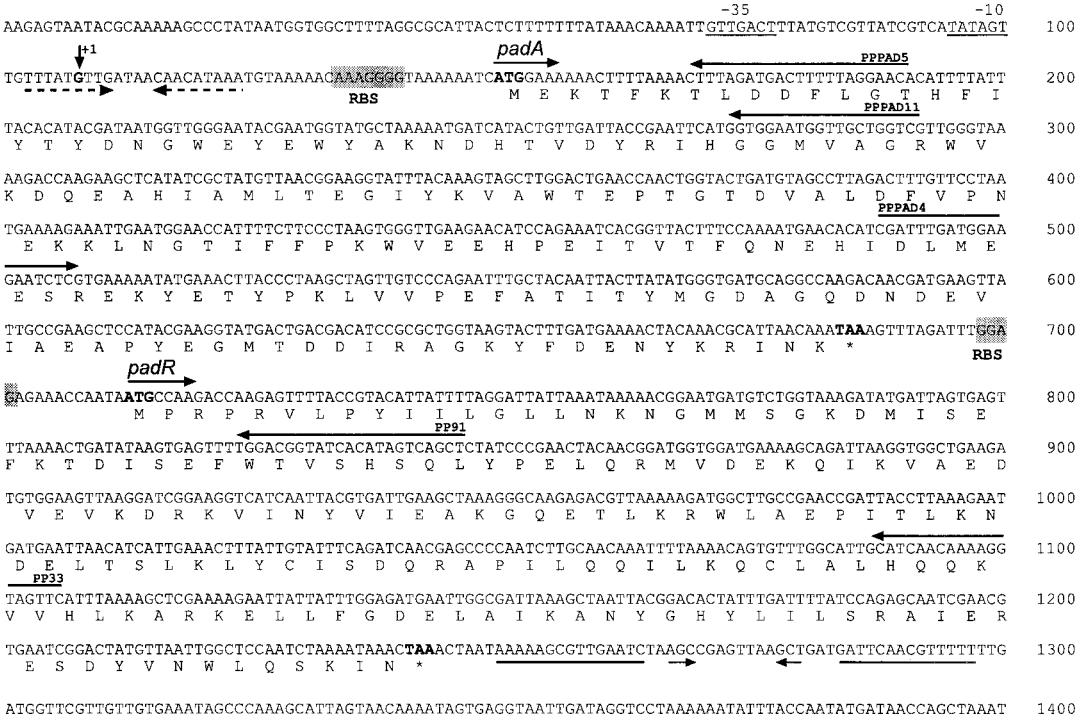

FIG. 2. Nucleotide and deduced amino acid sequences of the *pad* cluster. The localization and orientation of primers PPPAD5, PPPAD11, and PP91, used to identify the transcriptional start site of *padA* and *padR*, and primers PPPAD4 and PP33, used to amplify cDNA from *padA* mRNA, are indicated by horizontal arrows. The transcriptional start site of the *padAR* operon, determined by primer extension analysis, is indicated by a vertical arrow, and the corresponding −10 and −35 boxes are underlined. The putative ribosome binding sites (RBS) are shaded. Stop codons are indicated by asterisks. The two convergent arrows located under the sequence indicate the putative rho-independent transcriptional terminator of the *padAR* operon. The dotted convergent arrows indicate the region of dyad symmetry (inverted repeats), which could be the target of the PadR repressor.

sion product was generated when RNA from uninduced cells was used as the template. With RNA from induced cells, primer extension generated a band which identified a transcriptional start site identical to that of *padA* (data not shown). Taken together, these results clearly indicate that *padA* and *padR* are transcribed as a bicistronic unit and are subjected to transcriptional regulation which involves substrate-mediated induction.

Expression of PAD activity in *E. coli* is inhibited by the product or products of one or more genes present on pTPADP1. *E. coli* TG1(pTPADP1) conferred a PAD activity of 0.1 μmol · min^{-1} · mg^{-1} on *p*-coumaric acid, regardless of the presence of phenolic acid in the culture medium (data not shown). No PAD activity was found in the control strain *E. coli* TG1(pTZ19R). In addition, SDS-PAGE analysis of cell extracts from *E. coli* TG1(pTPADP1) and a control strain gave identical profiles (data not shown). These results clearly distinguish the *P. pentosaceus* recombinant PAD from the recombinant PDC of *L. plantarum*, which displays an inducible activity of 10 μmol · min^{-1} · mg^{-1} on *p*-coumaric acid, and the recombinant PAD from *B. subtilis*, which metabolizes *p*-coumaric, ferulic, and caffeic acids with a specific activity of about 2.5 μmol · min^{-1} · mg^{-1} in *E. coli*. Further hypotheses could explain the apparent low and constitutive activity conferred by the *P. pentosaceus padA* gene in *E. coli*: the *padA* promoter is poorly recognized by the *E. coli* RNA polymerase, the mRNA is unstable, the translation is low, the enzyme is unstable, and the regulation of PAD activity is somewhat altered in *E. coli*. To investigate the role of *padR*, plasmid pJPADP6 was constructed, which contained a 970-bp insert encompassing the *padA* gene and its promoter region, including 280 bp upstream of the 5′ end of the *padA* transcript (Fig. 1). Cell extracts of *E.*

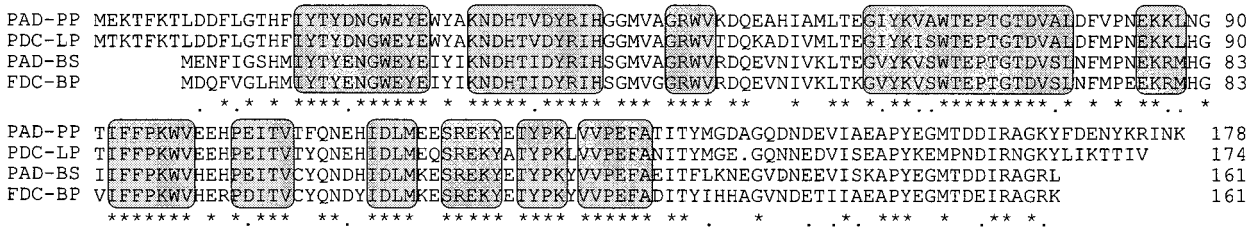

FIG. 3. Comparison of the deduced amino acid sequence of the *padA* gene of *P. pentosaceus* (PAD-PP) with the sequences of *B. pumilus* FDC (FDC-BP; accession no. X84815), *L. plantarum* PDC (PDC-LP; accession no. U63827), and *B. subtilis* PAD (PAD-BS; accession no. AF017117). The sequences were aligned by using the Clustal program. Identical and similar amino acids are indicated by asterisks and dots, respectively. Conserved boxes are shaded. The numbers on the right correspond to the amino acid position in the protein sequence.

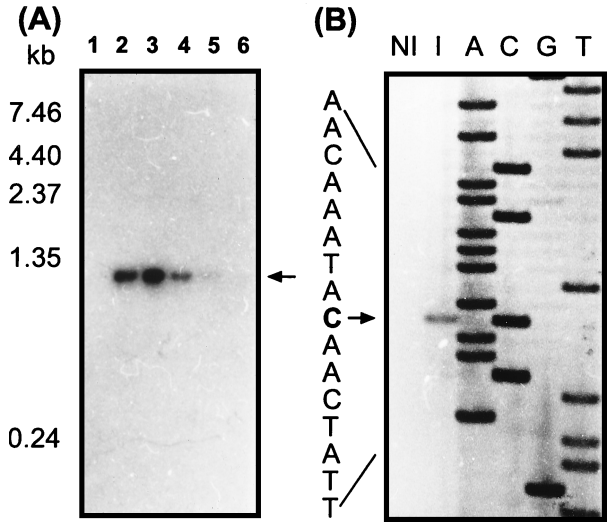

FIG. 4. Transcriptional analysis of the *padAR* operon. (A) Northern blot analysis with a *padA*-specific probe of total RNA purified from *P. pentosaceus* cells harvested after 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5), and 120 min (lane 6) following the addition of 2.4 mM *p*-coumaric acid. The arrow indicates the position of the transcript, and molecular size markers are given in the left lane. (B) Mapping of the 5′ end of the *padA* mRNA by primer extension analysis using primer PPPAD5 with total *P. pentosaceus* RNA from uninduced cells (NI) and cells induced with 2.4 mM *p*-coumaric acid (I). The products of reverse transcription were loaded in parallel with DNA sequencing reaction mixtures (lanes A, C, G, and T) initiated with the same primer on *padA* DNA template. The sequence shown to the left is the complementary strand, and the 5′ end of the *padA* mRNA is indicated by an arrow.

coli TG1(pJPADP6) and TG1(pJDC9) were prepared and analyzed by SDS-PAGE. A strong protein band of 25 kDa was detected in the recombinant *E. coli* strain carrying the *padA* gene and was absent in the control (Fig. 6). The observed protein band, which has a molecular mass corresponding to that of the deduced protein from the *padA* gene, correlated with a high PAD specific activity of 50 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ on *p*-coumaric acid, measured in cell extracts of TG1(pJPADP6). This activity was 500-fold higher than the maximal PAD activity found in *E. coli* TG1(pTPADP1) carrying the original insert. Our results indicate that the 6-kb pTPADP1 insert contains one or more genes involved in regulating the expression of PAD activity.

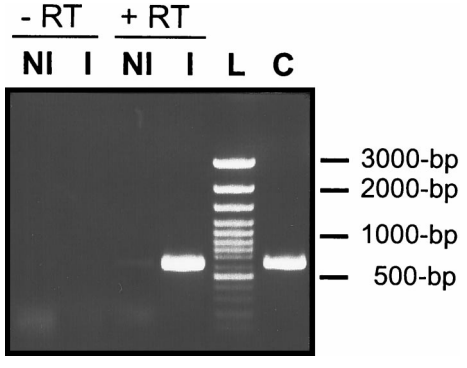

FIG. 5. RT-PCR of the *padAR* region using primers PPPAD4 and PP33 with *P. pentosaceus* total RNA purified from uninduced cells (NI) and cells induced with 2.4 mM *p*-coumaric acid (I). Negative controls with no RT (−RT) included are shown on the left. Classical PCR using the same primers and with *P. pentosaceus* chromosomal DNA added as a positive control is shown in lane C. The 100-bp DNA Ladder Plus (MBI Fermentas, Amherst, Mass.) was used as a molecular weight marker (L).

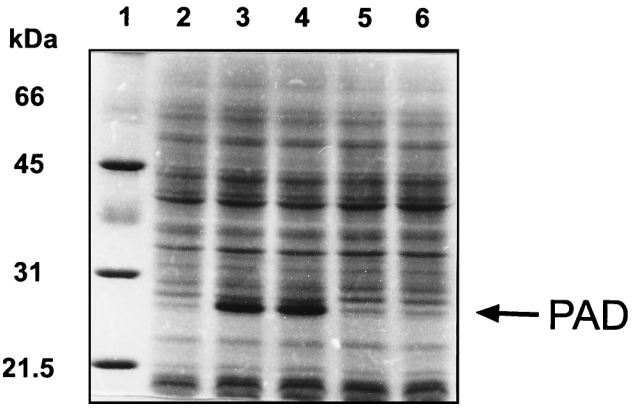

FIG. 6. SDS-PAGE of crude cell extracts from *E. coli* TG1 carrying various subclones of the *padAR* locus. Lanes: 1, molecular mass standards (SDS-PAGE standards; Bio-Rad); 2, crude extract from *E. coli* TG1(pJDC9); 3, crude extract from *E. coli* TG1(pJPADP6); 4, crude extract from *E. coli* TG1(pJPADP7); 5, crude extract from *E. coli* TG1(pJPADP8); 6, crude extract from *E. coli* TG1(pJPADP9). Molecular size markers are indicated on the left.

***padR* encodes a transcriptional repressor of the *pad* operon.** Two plasmids, pJPADP7 and pJPADP8, were constructed by amplifying pTPADP3 DNA as the template with PAD9 and U-primer, and PAD8 and R-primer, respectively (Fig. 1). Cell extracts from *E. coli* TG1(pJPADP8) exhibited a low PAD activity similar to that of *E. coli* TG1(pTPADP3), while *E. coli* TG1(pJPADP7) had a PAD activity 500-fold higher, identical to that of *E. coli* TG1(pJPADP6) (Fig. 1). Moreover, the PAD protein was detected by SDS-PAGE analysis in *E. coli* TG1(pJPADP7) cell extracts, but not in the cell extract from *E. coli* TG1(pJPADP8) (Fig. 6), indicating that the region downstream from *padA* was responsible for the low PAD activity. Plasmid pJPADP9 containing *padA* and *padR* was constructed (Fig. 1), and *E. coli* TG1(pJPADP9) cell extracts were analyzed by SDS-PAGE (Fig. 6) and PAD activity measurement (Fig. 1). The results indicate that PadR is responsible for the low PAD activity in *E. coli*.

We were unable to interrupt the chromosomal *padR* gene in *P. pentosaceus* due to the lack of electroporation or other transformation procedures for this species. To determine whether PadR could act as a transcriptional regulator in vivo, transcriptional analyses were carried out with the two *E. coli* clones containing pJPADP6 and pJPADP9. Total RNA was prepared from these two clones, and primer extension experiments were performed with primer PPPAD5 and with identical amounts of total RNA from both preparations. As shown in Fig. 7, the primer extension product obtained with total RNA from *E. coli* TG1(pJPADP9) was very weak compared to that obtained with *E. coli* TG1(pJPADP6), which had to be diluted 20-fold prior to loading onto the gel. The level of *padA* gene transcript was at least 1,000-fold higher in *E. coli* TG1(pJPADP6) than in TG1(pJPADP9), demonstrating that *padR* encodes a protein identified as a transcriptional repressor of the *padAR* operon.

DISCUSSION

Our studies indicate that the *P. pentosaceus* PadR protein represses transcription of the *padAR* operon, thereby regulating its own synthesis. Recently, we have cloned by random insertional mutagenesis, the *padR* gene from *B. subtilis* (J.-F.

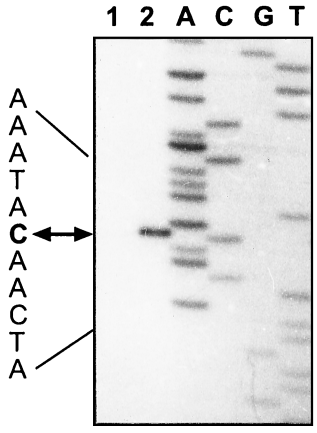

FIG. 7. Mapping of the 5′ end of *padA* mRNA by primer extension with primer PPPAD5 with total RNA from *E. coli* TG1(pJPADP9) (lane 1) and TG1(pJPADP6) (lane 2). The RT product from *E. coli* TG1(pJPADP6) was diluted 20-fold before loading. The products of reverse transcription reactions were loaded in parallel with DNA sequencing reaction mixtures (lanes A, C, G, and T) initiated with the same primer on the *padA* DNA template. The arrow indicates the 5′ end of the *padA* mRNA.

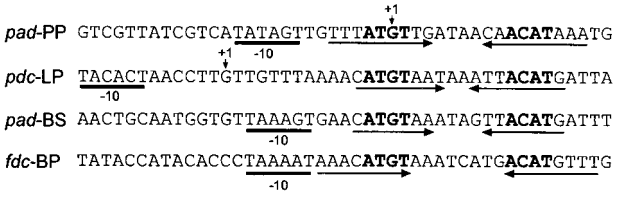

FIG. 8. Comparison of the promoter sequence from the *padAR* operon of *P. pentosaceus* (*pad*-PP) with the promoter sequences of the *L. plantarum pdc* gene (*pdc*-LP), *B. subtilis pad* gene (*pad*-BS), and *B. pumilus fdc* gene (*fdc*-BP). The putative −10 boxes are underlined. The transcription start sites are indicated by a vertical arrow, when determined. Convergent arrows indicate regions of dyad symmetry, and the conserved motif is highlighted in boldface.

Cavin, V. Dartois, and C. Diviès, unpublished data), which corresponds to a gene named *yfiO* in the *B. subtilis* genome sequence (28) and the deduced product of which displays the highest amino acid identity with PadR (37%). This last result is consistent with our finding and supports our results concerning the function of the PadR protein in *P. pentosaceus*.

To our knowledge, PadR is not a member of any known class of transcriptional regulators, but a search in public databases revealed significant homology with four different proteins, suggesting that we have identified a new class of bacterial regulatory proteins. One of them, RVI176C of *Mycobacterium tuberculosis*, is a protein of unknown function which displays 27% identity with PadR (15). The three other proteins have been identified as potential transcriptional regulators. OrfA of *Listeria monocytogenes* (27% amino acid identity with PadR) has been described as a putative negative regulator of the *hly* gene, coding for the pore-forming listeriolysin O, implicated in *L. monocytogenes* pathogenicity (26). The AphA protein of *Vibrio cholerae* (27% amino acid identity with PadR) is required for the expression of the ToxR virulence regulon and plays a role in the regulatory cascade that activates expression of the *tcpPH* operon (39).

Although the PADs of *P. pentosaceus*, *B. subtilis*, and *L. plantarum* (i) display a high amino acid identity of approximately 70%, (ii) exhibit similar activity levels on their respective substrates, and (iii) are all transcriptionally regulated, the genetic organization and regulation of their genes are distinct. The *padAR* genes of *P. pentosaceus* are transcribed as an operon, while the *padA* genes of *L. plantarum* and *B. subtilis* are monocistronic (8, 10). However, the *padR* gene of *B. subtilis* is not located in the vicinity of the *padA* gene, because 2,622 kbp separates the two genes (28). Identification of the *L. plantarum padR* gene is currently in progress in our laboratory. In *B. pumilus*, FDC expression was shown to be substrate regulated (16), but elucidation of *fdc* transcriptional regulation awaits further analyses. Interestingly, analysis of the nucleotide sequence of the *fdc* region (accession no. X84815) revealed a divergently transcribed 575-bp ORF located upstream from *fdc* and coding for a putative 185-amino-acid polypeptide which displays 57% identity with *B. subtilis* PadR and 36% identity with *P. pentosaceus* PadR.

The classical mode of action of known repressors, such as LacI in *E. coli* (1), involves binding as a dimer or a tetramer to specific DNA sequences which exhibit dyad symmetry. Analysis of the *P. pentosaceus padAR* promoter region revealed the existence of a perfect inverted repeat, TTTATGTTG-4N-CAACATAAA, which could be the target site for PadR binding. Interestingly, this motif was also partially found in the promoter region of all three *padA* genes from *L. plantarum*, *B. subtilis*, and *B. pumilus* (Fig. 8), where it is systematically located downstream from the −10 box near the transcription start site (8, 10, 41). It has been demonstrated that the position of the operator site within a promoter sequence determines the repression efficiency and that repressors which bind over the −10 box and start site occlude the most critical initiation region (34). Site-targeted modification of this conserved motif in the *P. pentosaceus padAR* promoter, combined with mobility gel shift assays and footprinting experiments using purified PadR, will be performed to identify the DNA binding site of PadR.

Inactivation of a repressor often involves a conformational change due to the direct binding of the inducer (2, 6). Although PAD activity was clearly induced by *p*-coumaric or ferulic acid in *P. pentosaceus*, addition of 3 mM *p*-coumaric or ferulic acid in a culture of *E. coli* TG1(pJPADP1), which displayed a low level of PAD, did not increase the level of decarboxylase activity. These results indicate either that phenolic acids remained unable to induce a conformational change of PadR in *E. coli*, or that phenolic acids act through an additional effector that is absent in *E. coli*. Such an effector could consist of a two-component system containing a sensor protein kinase, which would detect phenolic acids and activate a response regulator. Most of the time, the response regulator is a transcriptional activator (22). In some instances, however, information was shown to be transduced to a transcriptional repressor, as was demonstrated for LuxO in *Vibrio harveyi*, which is the final acceptor of a phosphorelay cascade and represses transcription of the *lux* operon in its phosphorylated form (23). Interestingly, only two systems induced by phenolic acids have been described in the literature: the two-component regulatory system involved in *vir* gene expression in *A. tumefaciens* and consisting of the VirA and VirG proteins (31), and the transcriptional activator PobR from *A. calcoaceticus* involved in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase (20).

Taken together, our results led us to propose a model for the regulation of the *padAR* operon in *P. pentosaceus* (Fig. 9). In the absence of phenolic acid in the medium, the PadR repressor binds to the region of dyad symmetry within the *padAR* promoter (Fig. 8). This prevents transcription of the *padAR* operon, leading to little or no PAD synthesis and to back-

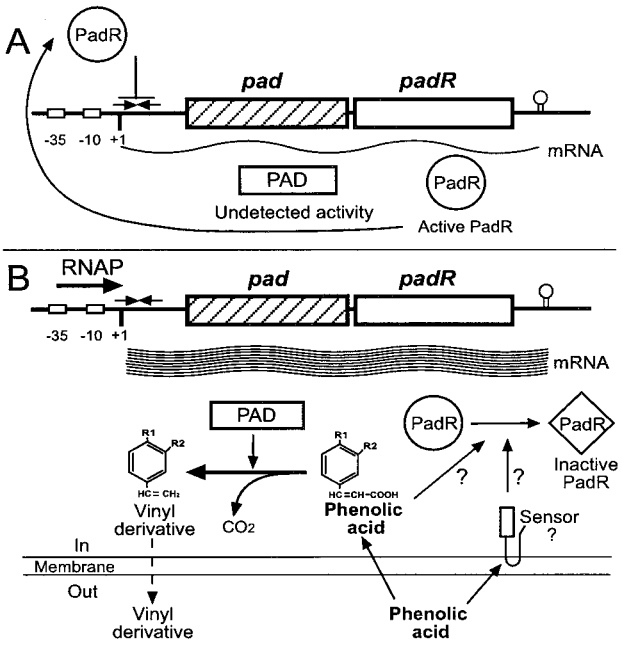

FIG. 9. Model for the transcriptional regulation of the *padAR* operon in *P. pentosaceus*. (A) In the absence of phenolic acid, the level of *pad* transcripts is low and could only be detected by RT-PCR, while PAD activity remained undetectable. (B) Addition of phenolic acid causes inactivation of the PadR repressor, possibly through an additional effector or sensor which mediates the conformational change or modulation of PadR. This allows transcription of the *padAR* operon and synthesis of the PAD enzyme. Toxic phenolic acids are decarboxylated into vinyl derivatives, which are less toxic and can diffuse outside the cell. Exhaustion of phenolic acids results in PadR switching to its active form and repressing *padAR* transcription. For *p*-coumaric acid, R1 = OH and R2 = H.

ground levels of PadR production. When phenolic acids are present in the medium, they induce inactivation of the PadR repressor by a mechanism which remains to be characterized. This allows transcription of the *padAR* operon and results in PAD and PadR synthesis. Thus, the PadR repressor remains in its inactive form as long as all available phenolic acids have not been converted by the PAD enzyme into 4-vinyl derivatives, compounds which are less toxic to lactic acid bacteria (4). When all available phenolic acid is degraded, PadR switches to the active form and turns off transcription of the *padAR* operon. The lactic acid bacterium *P. pentosaceus* seems to have evolved an efficient system to detoxify phenolic acids into 4-vinyl derivatives by organizing *padA*, which converts the toxic compound, and *padR*, which regulates PAD activity, into a single transcriptional unit. Genetic and biochemical studies are currently in progress in order to elucidate the mechanism of PadR inactivation by phenolic acids.

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