Knockout of the *p*-Coumarate Decarboxylase Gene from *Lactobacillus plantarum* Reveals the Existence of Two Other Inducible Enzymatic Activities Involved in Phenolic Acid Metabolism

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Lactobacillus plantarum NC8 contains a *pdc* gene coding for *p*-coumaric acid decarboxylase activity (PDC). A food grade mutant, designated LPD1, in which the chromosomal *pdc* gene was replaced with the deleted *pdc* gene copy, was obtained by a two-step homologous recombination process using an unstable replicative vector. The LPD1 mutant strain remained able to weakly metabolize *p*-coumaric and ferulic acids into vinyl derivatives or into substituted phenyl propionic acids. We have shown that *L. plantarum* has a second acid phenol decarboxylase enzyme, better induced with ferulic acid than with *p*-coumaric acid, which also displays inducible acid phenol reductase activity that is mostly active when glucose is added. Those two enzymatic activities are in competition for *p*-coumaric and ferulic acid degradation, and the ratio of the corresponding derivatives depends on induction conditions. Moreover, PDC appeared to decarboxylate ferulic acid in vitro with a specific activity of about $10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the presence of ammonium sulfate. Finally, PDC activity was shown to confer a selective advantage on LPNC8 grown in acidic media supplemented with *p*-coumaric acid, compared to the LPD1 mutant devoid of PDC activity.

Substituted hydroxycinnamic acids (principally ferulic acid and *p*-coumaric acid), also called phenolic acids, are abundant molecules that bind the complex lignin polymer to the hemicellulose and cellulose in plant cell walls (16). Various bacteria and fungi produce a wide range of hemicellulases in order to degrade these cell wall polymers (17) and to release phenolic acids, which are biologically important molecules. First, they serve as signals for the plant-associated *Agrobacterium tumefaciens* and induce *vir* gene expression through a two-component system consisting of the VirA and VirG proteins (28). The Vir-inducing properties of ferulic acid in *A. tumefaciens* were shown to be strain dependent (29), while ferulic acid could be O-demethylated into caffeic acid by the VirH2 protein in order to turn off *vir* gene expression (26).

Phenolic acid catabolism is also essential in the biodegradation of plant wastes. Several bacteria, such as *Pseudomonas* spp. and *Acinetobacter calcoaceticus*, are able to grow on these compounds as the sole source of carbon. In the first step, they convert ferulic and *p*-coumaric acids into vanillic and *p*-hydroxybenzoic acids, respectively, which are then transformed into protocatechuic acid and integrated into the tricarboxylic acid cycle via the β -ketoadipate pathway (33, 38, 41). Ferulic acid can also be degraded into vanillin by a two-step process involving either a coenzyme A (CoA) ligase followed by side chain cleavage in *Pseudomonas fluorescens* (21) and *Pseudomonas* sp. strain HR199 (34) or a propionic acid chain cleavage followed by a reductase in the white-rot fungus *Pycnoporus cinnabarinus* (2, 31). In other microbial systems, phenolic acids are metabolized into volatile phenols by two different pathways. Most often, they are first decarboxylated into 4-vinyl derivatives and then reduced to 4-ethyl derivatives. Phenolic

acid decarboxylases (PAD) have been characterized in yeast and bacteria (7, 19). Four genes coding for PAD enzymes, *pad1* from *Saccharomyces cerevisiae* (13), *fdc* from *Bacillus pumilus* (44), *pdc* from *Lactobacillus plantarum* (7), and *pad* from *Bacillus subtilis* (9) have been cloned and expressed in *Escherichia coli*. Recently, several gene products sharing significant homology with PAD1 from *S. cerevisiae* have been discovered through genome sequencing; these are encoded by *pad1* from *Archaeoglobus fulgidus* (27), *pad1* from *E. coli* (accession no., AJ006210), *vdcb* from *Streptomyces* sp. strain D7 (12), *yclB* from *B. subtilis* (43), and MJ0102 from *Methanococcus jannaschii* (4), although the corresponding enzymatic activities have not been demonstrated so far. A second pathway has been proposed for *Lactobacillus pastorianus* where caffeic and *p*-coumaric acids are first reduced into substituted phenyl propionic acids and then decarboxylated into 4-ethyl derivatives (42).

The interest in improving our understanding of phenolic acid biodegradation is multiple. First, as was shown for *S. cerevisiae*, PAD activity may confer a selective advantage upon microorganisms during growth on plants, where PAD expression could constitute a stress response induced by phenolic acid (13, 23). Second, phenol derivatives are valuable intermediates in the biotechnological production of new flavor and fragrance chemicals (25). Third, they are regarded as sources of phenolic off-flavors in many beers and wines, due to their characteristic aroma and their low threshold of detection (20, 39).

L. plantarum is now considered a model for ubiquitous lactic acid bacteria in the plant kingdom. Moreover, it is used as a malolactic starter in some wines and as a lactic starter for many vegetable fermentations which contain phenolic acids. *L. plantarum* displays substrate-inducible PAD activity encoded by the *pdc* gene, which is transcriptionally regulated by *p*-coumaric, ferulic, and caffeic acids (7). Under the conditions tested, the purified *p*-coumaric acid decarboxylase (PDC) exhibits high

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i> TGI	<i>supE hsdΔ5thi Δ(lac-proAB)F'</i> [<i>traD36 proAB⁺ lac^q lacZΔM15</i>]	22
<i>L. plantarum</i> CHL2	Wild-type, gram-positive, ubiquitous homolactic acid bacterium	Charles Hansen's BioSystems, Horsholm, Denmark
<i>L. plantarum</i> NC8	Plasmid-free strain	L. Axelsson, presented at the Fifth Symposium on Lactic Acid Bacteria, Veldhoven, The Netherlands, 1996.
Plasmids		
pBluescript SK(−)	Ap ^r <i>ΔlacZ</i>	Stratagene
pGID023	Shuttle vector for <i>E. coli</i> and <i>L. plantarum</i> ; derivative of pJDC9 containing the pE194 replication functions; used as an unstable integration vector; Em ^r	24
pBSK [−] FA	Ap ^r ; pBluescript SK(−) containing the 537-bp fragment (FA) of <i>L. plantarum</i> amplified by PCR with primers D1 and D2	This study
pBSK [−] FAC	Ap ^r ; pBluescript SK(−) containing FA and the 497-bp (FC) fragment of <i>L. plantarum</i> amplified by PCR with primers D3 and D4	This study
pGFAC	Em ^r ; pGID023 containing the 1,034-bp FAC fragment	This study
pJDC9	Em ^r ; <i>ΔlacZ</i>	11
PJPDC1	pJDC9 containing the 2.3-kb <i>Sau3A</i> fragment of <i>L. plantarum</i> CHL2 with the <i>pdC</i> gene	7

specific activity on *p*-coumaric acid ($0.6 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) but not detectable activity on ferulic acid (8).

We have constructed an *L. plantarum* mutant strain deficient in PDC activity in order to investigate alternate pathways for phenolic acid degradation and to study the stress response. Kinetic studies of the *pdC* mutant revealed the existence of a second PDC activity, called PDC2, as well as an alternate phenolic acid reduction pathway, in *L. plantarum*. Our results also suggest that the synthesis of PDC in *L. plantarum* could constitute a stress response induced by phenolic acid toxicity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. plantarum* LPNC8 (kindly provided by Lars Axelsson), LPCHL2, and LPD1 were grown at 37°C in MRS medium (15) with glucose (20 g/liter) as the source of carbon. *E. coli* TG1 was grown at 37°C on Luria-Bertani (LB) medium (3). Antibiotics were used in the following concentrations: erythromycin at 200 mg/liter for *E. coli* and 5 mg/liter for *L. plantarum* and ampicillin at 200 mg/liter for *E. coli*.

PCR amplification of DNA. To construct the *L. plantarum* *pdC* mutant, the plasmid-free strain LPNC8 was preferred to LPCHL2, which carries two plasmids, in order to avoid incompatibility between any of these plasmids and the unstable autoreplicative vector pGID023. To verify that the *pdC* gene locus was identical in LPCHL2 and LPNC8, Southern blotting and DNA sequencing of the *pdC* gene region were carried out in both strains. *L. plantarum* NC8 chromosomal DNA was used to perform PCR amplification. Primers D1 (5' AGCCTGCAG ACCGACACTGATCCACTC 3') and D2 (5' AGCGATATCGACCAACGA CCGGCACC 3'), which include *Pst*I and *Eco*RV sites, respectively, (underlined nucleotides), were used to amplify the FA region, which corresponds to a 537-bp DNA fragment of the *pdC* locus encompassing the putative promoter region and the first 152 nucleotides of the open reading frame. Primers D3 (5' AGCGAT ATCGTCTCGTGAAAAGTATGCC3') and D4 (5' GGCAAGCTTGCAGAG CAAGGTAAG 3'), which include *Eco*RV and *Hind*III sites, respectively (underlined nucleotides), were used to amplify the FC region, which corresponds to a 497-bp DNA fragment containing the last 174 nucleotides of the *pdC* open reading frame followed by a putative transcriptional terminator (Fig. 1). A 160-bp *pdC* internal fragment, located within the deleted region, was amplified with primers R1 (5' GCTGACATCGTCATGTTG 3') and R2 (5' GTTTCCTTAAATCGATG 3'). PCR amplification was performed using 0.1 μg of DNA template with 0.5 U of *Taq* DNA polymerase (Appligene) under standard conditions in an automatic DNA thermocycler (Hybaid, Ltd., Teddington, United Kingdom).

DNA manipulation and transformation procedures. Standard procedures described by Sambrook et al. (36) were used for DNA manipulation. *L. plantarum* chromosomal DNA was prepared by the method described by Posno et al. (35). PCR products were purified with the Jet Pur Kit (Q.BIOgene, Illkirch, France) and sequenced by the dideoxy chain termination method (37) with the Thermo-sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Inc., Cleveland, Ohio) in accordance with the recommendations of the

manufacturer. Southern blotting was performed as previously described (7). The 160-bp PCR product was labeled with 10 μCi of [α - ^{32}P]dATP (NEN, Boston, Mass.) by 10 PCR amplification cycles with primers R1 and R2 to generate a highly radiolabeled double-stranded probe. *E. coli* and *L. plantarum* strains were transformed by electroporation as described by Dower et al. (18) and Aukrust and Nes (1), respectively.

Preparation of whole-cell suspensions and cell extracts. Cells of *L. plantarum* grown in MRS medium and *E. coli* grown in LB medium were harvested by centrifugation, washed twice with 25 mM potassium phosphate buffer (pH 6.0), and resuspended in the same buffer. For low-activity detection (a few nanomoles per minute per milligram), concentrated resting cells (5 g/liter [dry weight]) were used in kinetic reactions. For high-activity detection, diluted resting cells (0.2 g/liter [dry weight]) were used. For cell extract preparation, cells were harvested as described above and disrupted with a French press at 1.2×10^8 Pa. The total protein concentration in the cell extract was determined by using a protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard. The specific activity was expressed as micromoles or nanomoles of substrate degraded per minute per milligram of protein. For whole cells, the protein concentration was deduced from the dry biomass in the cell suspension (1 g of dry biomass per liter corresponds to 0.5 g of total protein per liter).

Assay of phenolic acid degradation. Phenolic acid degradation and derivative production were monitored by UV spectrophotometry (using quartz cuvettes in a Beckman DU600 spectrophotometer). The products identified by UV spectrophotometry (phenolic acids and phenolic derivatives) have been previously identified by gas chromatographic analysis (6) and also by high-pressure liquid chromatographic (HPLC) analysis (14). UV spectrophotometry has since been used

FIG. 1. Physical map of the *pdC* locus in the wild-type strain LPNC8 (a) and the mutant strain LPD1 (b). Long horizontal arrows represent the two ORFs and their orientations. The start sites are indicated by vertical arrows and the stop codons by T. The positions and orientations of the primers are indicated by short horizontal arrows, and restriction sites that were created are noted between brackets.

FIG. 2. UV spectra of *p*-coumaric acid and metabolic derivatives: 1, 60 μ M *p*-coumaric acid; 2, 60 μ M 4-vinyl phenol; 3, 60 μ M phloretic acid; 4, 60 μ M 4-ethyl phenol; 5, 30 μ M 4-vinyl phenol with 30 μ M phloretic acid. Arrows point to the maximum absorbance for each compound.

to study phenolic acid metabolism, taking advantage of the rapidity, sensitivity, and reliability of this method (7, 9, 14). *p*-coumaric acid has a main absorption peak at 285 nm and a second, lower peak at 305 nm. Ferulic acid has two absorption peaks, at 285 and 300 nm. Decarboxylation and reduction of phenolic acids lead to a hypsochrome UV spectrum displacement, as was shown for the *p*-coumaric acid derivatives 4-vinyl phenol, 4-ethyl phenol, and phloretic acid (Fig. 2). Analysis of phenolic acid metabolism could be performed using this method in different reaction mixtures. In MRS medium, the sample has to be centrifuged first and the supernatant is diluted 100-fold prior to UV analysis. Ferulic and *p*-coumaric acid degradation could be detected at 300 and 305 nm, respectively, as these two acids have a second absorption peaks at these wavelengths. Detection of the phenolic acid derivatives is not possible because components of MRS medium interfered with the UV spectra in a range of 255 to 280 nm. In order to identify the phenolic acid degradation products, analyses were carried out with cell extracts or with whole cells in phosphate buffer. The absence of UV absorbance of the phosphate buffer and Stop buffer (20 mM Tris-HCl-0.3% sodium dodecyl sulfate [SDS] to stop activity; pH 6.0) used in these experiments was first checked. Moreover, the stability of phenolic acids in the phosphate buffer was also verified in the time of the experiments. Whole cells of *L. plantarum* were incubated in phosphate buffer to check that this bacterium did not produce extracellular proteins which could interfere with UV analysis. Kinetic reactions with cell extracts (about 5 to 50 mg of proteins/liter) in phosphate buffer were started by adding 0.6 mM substrate. During incubation, samples were taken and diluted 50-fold in Stop buffer prior to UV analysis. Nevertheless, low-specific-activity detection by UV spectrophotometry was critical in cell extracts, since high protein concentrations have to be incubated with relatively small amounts of substrate (0.06 to 0.3 mM). Under these conditions, UV spectra are disturbed by UV absorbance of soluble proteins, and the appearance of 4-vinyl phenol or other derivatives may remain undetected. Since the uptake of phenolic acids into bacterial cells is not a limiting step for their metabolism, the problem of low-specific-activity detection was solved by running kinetic experiments in phosphate buffer using highly concentrated whole-cell suspensions. In this case, whole-cell suspensions were prepared as described above. Reactions were started by adding 0.6 mM substrates. Samples were taken and were immediately centrifuged (at 4°C, for 15 min, at 12,000 $\times$ g) to eliminate the cells. The supernatant was diluted 50-fold in Stop buffer before UV spectrophotometry analysis.

UV spectrophotometry analysis is suitable for detecting low phenolic compound concentrations (1 to 10 μ M). As kinetic reactions are most often performed with 1.2 or 0.6 mM phenolic acids, the dilution of the sample before UV analysis strongly reduces the UV absorbance of the mixture reaction components when necessary (for samples from MRS medium). In all cases, the UV spectra obtained under these conditions have no background signals.

RESULTS

Cloning of a deleted copy of the *pdc* gene. The FA and FC regions of the *pdc* gene (Fig. 1) were amplified separately by PCR and used for the construction of plasmid pGFAC. The FA fragment was cloned into the pBluescript SK(−) vector between the *Pst*I and *Eco*RV sites. The FC fragment was then ligated into pBSK[−]FA between *Eco*RV and *Hind*III in the same transcriptional orientation. The resulting plasmid, named

pBSK[−]FAC, bears a copy of the *pdc* gene lacking 199 bp of its internal region. A frameshift in the deleted gene copy downstream from the *Eco*RV site creates a stop codon, which causes the synthesis of a truncated peptide of 51 amino acids (Fig. 1). The 1,034-bp *Pst*I/*Hind*III fragment from pBSK[−]FAC was cloned into the shuttle vector pGID023 to give plasmid pGFAC, which was transformed into *E. coli* TG1. Whole cells and corresponding cell extracts of *E. coli* TG1(pGFAC) were prepared as described in Materials and Methods and were tested for their ability to metabolize *p*-coumaric and ferulic acids (data not shown). No PDC activity was detected on either substrate, even after 24 h of incubation, while whole cells and cell extracts of *E. coli* TG1(pJPDC1), containing the wild-type *pdc* gene, exhibited PDC activity of 8.5 μ mol $\cdot$ min^{−1} $\cdot$ mg^{−1} on *p*-coumaric acid (7). These results indicated that plasmid pGFAC was suitable for generating a *pdc* mutant strain of *L. plantarum* by gene replacement.

Disruption of the *pdc* gene in LPNC8. Plasmid pGFAC was introduced in LPNC8 by electroporation, and transformants were selected for erythromycin resistance. The primary recombination event between pGFAC and the chromosomal *pdc* locus led to integration of the whole plasmid and thus conferred erythromycin resistance. The second recombination event, between the chromosomal *pdc* gene and its deleted copy, followed by segregational loss of the excised vector, produced erythromycin-sensitive clones. Since the excision step can generate either the wild-type PDC phenotype or a deletion in the chromosomal *pdc* gene, PCR analyses on total DNA of erythromycin-sensitive candidates (Em^s) were carried out to screen for clones bearing the deleted *pdc* gene copy. Two primers located on each side of the deleted region were used, which should result in amplification of a 540-bp fragment for the wild-type gene and of a 342-bp fragment for the deleted gene. Among 10 Em^s colonies tested, four clones, designated D1, D2, D8, and D10, yielded the shorter PCR fragment, while all others, as well as the wild-type control LPNC8, gave the 540-bp fragment of the full-length gene. Sequencing of the 342-bp PCR fragment confirmed the deletion (data not shown). Southern blotting was performed on total DNA of D1, D2, D8, and D10, which was then digested by *Eco*RI/*Hind*III and hybridized with a 160-bp labeled probe located in the deleted region. Positive hybridization was obtained with the wild-type LPNC8 strain, while no hybridization could be detected with clones D1, D2, D8, and D10 (data not shown). These results further confirmed that clones D1, D2, D8, and D10 carried a 199-bp internal deletion in the *pdc* gene. One of the latter clones, designated LPD1, was retained for further studies.

Ability of an LPD1 mutant to metabolize *p*-coumaric acid. Wild-type LPNC8 and the LPD1 *pdc* mutant were grown in MRS medium alone or supplemented with 1.2 mM *p*-coumaric acid. Samples were taken during the growth cycle to measure the *p*-coumaric acid concentration by UV spectrophotometry at 305 nm, in order to avoid interference with MRS components. The two strains displayed similar growth curves with and without *p*-coumaric acid but showed different rates of *p*-coumaric acid degradation (Fig. 3). LPNC8 degraded 100% of available *p*-coumaric acid within the first 2.5 h of growth, while no *p*-coumaric acid appeared to be degraded during the same period in the LPD1 culture. However, the LPD1 mutant started to weakly metabolize *p*-coumaric acid after 5 h, and all available *p*-coumaric acid had disappeared from the medium after 10 h. This indicated that LPD1 still produced one or more enzymes, distinct from PDC, responsible for *p*-coumaric acid utilization. Phenolic acid pathway analysis was performed using whole cells and cell extracts in phosphate buffer to elimi-

FIG. 3. Growth of strain LPNC8 (squares) and the LPD1 mutant (circles) supplemented (filled symbols) or not (open symbols) with 1.2 mM *p*-coumaric acid at pH 6.5. Residual *p*-coumaric acid concentrations in LPNC8 (filled triangles) and in LPD1 (open triangles) were measured by UV spectrophotometry (see Materials and Methods).

nate absorption in the UV range by MRS components. LPNC8 and LPD1 were grown in MRS medium and divided in two subcultures. One subculture was used as a control and was incubated for 2 h at 37°C with no addition, while the other was supplemented with 3 mM ferulic acid and incubated under the same conditions. Whole cells and cell extracts prepared from these subcultures were tested for *p*-coumaric acid degradation activity. No acid degradation was detected in the uninduced whole cells and cell extracts of either strain. In LPNC8, the wild-type strain, *p*-coumaric acid was decarboxylated by whole induced cells and corresponding cell extracts with an activity of $500 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. In addition, *p*-coumaric acid was also decarboxylated into 4-vinyl phenol by the LPD1 mutant, but at a lower rate of about $5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, indicating that a second PAD was synthesized by LPD1. The PDC activity of LPD1 was 100-fold lower than that of LPNC8, the wild-type strain, and was for this reason totally concealed by the high PDC activity conferred by the *pdc* gene. In the LPD1 cell extract, this second PAD activity was not detected. Studies of phenolic acid metabolism were therefore carried out with whole cells of LPNC8 and LPD1.

Evidence of a second PDC enzyme and a phenolic acid reductase in *L. plantarum*. In order to characterize the second PAD activity, growing cultures of LPD1 and LPNC8 were divided into four samples and induced with 1.2 or 3 mM *p*-coumaric acid or ferulic acid. Whole resting cell suspensions were prepared in 25 mM phosphate buffer (pH 6.0) in order to identify *p*-coumaric acid degradation products (Table 2). In the wild-type strain, LPNC8, UV spectra indicate that *p*-coumaric acid was strongly decarboxylated into 4-vinyl phenol with a specific activity of $500 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (Fig. 4a). In LPD1 mutant, *p*-coumaric acid metabolism was dependent on the nature and concentration of the inducer. Cells induced with ferulic acid (1.2 or 3 mM) displayed *p*-coumaric acid decarboxylation activity of about $8 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. In cells induced with 1.2 mM *p*-coumaric acid, *p*-coumaric acid was metabolized but 4-vinyl phenol was not the product of the reaction. Instead, phloretic acid or 4-ethyl phenol appeared to be produced, based on the UV spectrum (Fig. 4b). For cells induced with 3 mM *p*-coumaric acid, UV spectra indicate that *p*-coumaric acid was degraded into a mixture of 4-vinyl phenol and phloretic acid or 4-ethyl phenol (Fig. 4c). The production

TABLE 2. Derivatives accumulated upon biotransformation of *p*-coumaric acid by whole cells of LPNC8 and the LPD1 mutant, induced with *p*-coumaric or ferulic acid

Strain	Derivative(s) present after induction ^a by:							
	<i>p</i> -Coumaric acid				Ferulic acid			
	1.2 mM		3 mM		1.2 mM		3 mM	
	-	+	-	+	-	+	-	+
LPNC8	4VP	4VP	4VP	4VP	4VP	4VP	4VP	4VP
LPD1	RD	RD	4VP/RD	4VP	4VP	RD	4VP	4VP/RD

^a Whole cells were incubated for 15 min at 30°C with (+) or without (-) 20 mM glucose before kinetic reactions. 4VP, 4-vinyl phenol; RD, reduced derivatives, i.e., phloretic acid and/or 4-ethyl phenol; 4VP/RD, mixture of 4-vinyl phenol and reduced derivatives.

of 4-ethyl derivatives from phenolic acids requires the prior formation of 4-vinyl derivatives or substituted phenyl propionic acids (7). As 4-vinyl derivatives were never detected during kinetic experiments with LPD1 cells, phenolic acids were likely reduced into substituted phenyl propionic acids and subsequently decarboxylated into 4-ethyl derivatives. These results suggested that LPD1 could produce a phenolic acid reductase, which metabolized *p*-coumaric acid into phloretic acid, as was shown for *L. pastorianus* (42). Since a reduced cofactor is generally required for enzymatic reduction (30, 32), similar experiments were done to confirm the hypothesis of reduction, using whole cells incubated for 15 min with 20 mM glucose at 30°C prior to starting the kinetics, in order to stimulate glycolysis and increase the pool of reduced cofactors. No difference was detected in the wild-type strain, while the addition of glucose in whole cells of LPD1 strongly stimulated the transformation of *p*-coumaric acid into a phenol derivative which was not 4-vinyl phenol but was phloretic acid and/or 4-ethyl phenol, for cells induced by *p*-coumaric acid (1.2 and 3 mM) and 1.2 mM ferulic acid (Table 2). Only LPD1 cells induced with 3 mM ferulic acid still produced a mixture of 4-vinyl phenol and phloretic acid and/or 4-ethyl phenol (Table 2). Taken together, our results indicate the presence, in both wild-type and LPD1 strains, of a second PAD (named PDC2), highly induced by ferulic acid, and of a putative phenolic acid reductase activity (named PAR) induced by *p*-coumaric and ferulic acids in the presence of glucose. These two enzymes displayed low specific activities (about $10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for *p*-coumaric acid) compared to PDC.

FIG. 4. UV spectra resulting from the conversion of 0.6 mM *p*-coumaric acid by induced whole cells of LPNC8 (0.2 g/liter) or LPD1 (5 g/liter) incubated 1 h without glucose in 25 mM phosphate buffer. *t*₀, UV spectra corresponding to the sample taken at the start of kinetic reaction. *t*₁, UV spectra corresponding to the sample taken after 1 h of kinetic reaction. (a) LPNC8 induced with *p*-coumaric or ferulic acid (1.2 or 3 mM); (b) LPD1 induced with 1.2 mM *p*-coumaric acid; (c) LPD1 induced with 3 mM *p*-coumaric acid.

TABLE 3. Derivatives from the biotransformation of ferulic acid by whole cells of LPNC8 and the LPD1 mutant, induced with *p*-coumaric or ferulic acid

Strains	Derivative(s) present after induction ^a by:							
	<i>p</i> -Coumaric acid				Ferulic acid			
	1.2 mM		3 mM		1.2 mM		3 mM	
	–	+	–	+	–	+	–	+
LPNC8	4VG	4VG/RD	4VG	4VG/RD	4VG	4VG/RD	4VG	4VG
LPD1	RD	RD	4VG/RD	RD	4VG	RD	4VG	4VG/RD

^a Whole cells were incubated for 15 min at 30°C with (+) or without (–) 20 mM glucose before kinetic reactions. 4VG, 4-vinyl gäiäcol; RD, reduced derivatives, i.e., dihydroferulic acid and/or 4-ethyl gäiäcol; 4VG/RD, mixture of 4-vinyl gäiäcol and reduced derivatives.

The PDC enzyme displays weak decarboxylase activity on ferulic acid. Whole resting cell suspensions of LPD1 and LPNC8 induced with *p*-coumaric or ferulic acid were prepared as previously described and tested for ferulic acid metabolism. Ferulic acid derivatives produced by whole cells of LPD1 were different from those produced by whole cells of LPNC8. Ferulic acid was partially or totally reduced, depending on the nature and the concentration of the inducers, by the LPD1 mutant, but was preferentially decarboxylated in the wild-type strain, LPNC8 (Table 3). This indicates that the PDC enzyme is involved in ferulic acid decarboxylation in whole cells. To confirm this hypothesis, which is contradictory to the results obtained previously with the purified PDC (8), ferulic acid metabolism was tested in whole cells of the recombinant strain *E. coli* TG1(pJPDC1) overexpressing the PDC enzyme (7) and on the corresponding cell extract. In whole cells, ferulic acid was decarboxylated at a rate of about 40 nmol · min^{–1} · mg^{–1}, while the corresponding cell extract displayed no detectable activity on ferulic acid in phosphate buffer. Reaction conditions were then modified by varying the pH and temperature independently and by adding glycerol or salts. Ferulic acid decarboxylase activity was stimulated in cell extracts supplemented with 20% ammonium sulfate or 20% NaCl, with an optimum activity of about 30 nmol · min^{–1} · mg^{–1}, indicating that PDC displays a low ferulic acid decarboxylase activity under those conditions.

Influence of the *p*-coumaric acid concentration on the growth of the wild-type strain, LPNC8, and the mutant LPD1 strain at different pHs. Three concentrations of *p*-coumaric

acid (0.6, 3, and 6 mM) were tested on the growth of the LPNC8 and LPD1 strains in MRS broth at pH 6.5. The residual *p*-coumaric acid concentration was measured during growth. For the wild-type strain, LPNC8, addition of 0.6 or 3 mM *p*-coumaric acid in the culture medium had no apparent effect on growth (Fig. 5a). The totality of this acid was degraded in, respectively, 2 or 3 h. Addition of 6 mM *p*-coumaric acid increased the latency period, but when all the available *p*-coumaric acid was metabolized, the final biomass was the same as that of the control culture without *p*-coumaric acid (data not shown). In contrast to results for the wild-type strain, *p*-coumaric acid was not degraded during the first 5 h of LPD1 mutant growth (Fig. 5b). With 0.6 mM *p*-coumaric acid, LPD1 metabolized 100% of this acid after 7 h of growth and its growth was not inhibited. For cultures with 3 and 6 mM *p*-coumaric acid, only 80 and 90%, respectively, was metabolized in the same period. LPD1 growth was significantly decreased, and the final biomass reached only 78 and 57% compared to that of the control without acid (data not shown). These results seem to indicate that *p*-coumaric acid is toxic for *L. plantarum*. The entrance by diffusion of weak acids into bacterial cells increases with a decrease in external pH (45). In order to observe the influence of different rates of acid uptake on the growth, cultures were performed at different initial pHs (5.5, 4.5, and 3.5) in MRS medium and at three *p*-coumaric acid concentrations (1.2, 3, and 6 mM). The most significant results were obtained at pH 4.5. The growth of the wild-type strain, LPNC8, was not modified by 1.2 or 3 mM *p*-coumaric acid and was only reduced with 6 mM (Fig. 6a). The growth of the LPD1 mutant

FIG. 5. Growth of (filled symbols) and degradation of *p*-coumaric acid by (open symbols) LPNC8 (a) and LPD1 (b) at different *p*-coumaric acid concentrations (◆, 0 mM; ■, 0.6 mM; ●, 3 mM; ▲, 6 mM) at pH 6.5. Samples were taken during growth to determine the biomass (OD₆₀₀) and *p*-coumaric acid degradation using UV spectrophotometry (see Materials and Methods).

FIG. 6. Growth of LPNC8 (a) and LPD1 (b) at different *p*-coumaric acid concentrations (◆, 0 mM; ■, 1.2 mM; ●, 3 mM; ▲, 6 mM) at pH 4.5.

strain was strongly inhibited in the presence of 1.2 or 3 mM *p*-coumaric acid and was totally inhibited with 6 mM *p*-coumaric acid (Fig. 6b). These results indicate that *p*-coumaric acid toxicity was higher at a low initial pH of the growth medium.

DISCUSSION

Inactivation of the *pdc* gene revealed some interesting features of phenolic acid metabolism in *L. plantarum*. Most notable was a low residual PAD activity in the LPD1 mutant, attributed to an alternate PDC2 activity that weakly decarboxylates *p*-coumaric and ferulic acids into 4-vinyl derivatives and is better induced with ferulic acid. This is, to our knowledge, the first report which demonstrates the existence of two distinct and functional acid phenol decarboxylases in a single microorganism.

Our study also reveals that *L. plantarum* converts phenolic acids into substituted phenyl propionic acids. Therefore, *L. plantarum* appears to have *p*-coumaric and ferulic acid reductase activities (PAR), induced by both substrates and mostly active when glucose is added (Tables 2 and 3). Reduction of *p*-coumaric and caffeic acid has been previously demonstrated in *L. pastorianus* (42). In *L. plantarum*, PAR and PDC2 activities are in competition for *p*-coumaric and ferulic acid degradation, and the ratio of the corresponding derivatives depends on induction conditions. The stable food grade mutant LPD1 strain, which was obtained by a two-step homologous recombination event, is devoid of its major PAD activity. However, it is still able to metabolize phenolic acids, and it is not suitable for producing fermented food or beverages without phenolic acid derivatives.

In a previous work (8), the existence of the second PAD, PDC2, was supported by the facts that ferulic acid was decarboxylated by whole cells of *L. plantarum* and that the purified PDC from *L. plantarum* did not display activity on ferulic acid under the conditions tested. The present results nevertheless indicate that PDC exhibits a weak decarboxylase activity in vitro on ferulic acid when ammonium sulfate or NaCl are added in the reaction buffer. This constitutes the major difference between *L. plantarum* PDC and both ferulic acid decarboxylase (FDC) from *B. pumilus* (44) and PAD from *B. subtilis* (9), which metabolize *p*-coumaric and ferulic acids in vivo and in vitro at similar rates, without salt addition. The FDC and PAD enzymes display strong amino acid sequence identity (88%), while PDC shares only 62 and 66% identity with FDC and PAD, respectively, with the most diverging domains being located in the N- and C-terminal protein regions. Construction

of chimeric proteins is currently in progress in order to correlate the protein domain(s) with the substrate specificity and metabolic characteristics of these enzymes.

We have demonstrated that *L. plantarum* has three inducible activities for the degradation of *p*-coumaric and ferulic acids (Fig. 7), which we think may be involved in the stress response induced by phenolic acids. The functional PDC enzyme clearly confers a selective advantage on the wild-type strain, LPNC8, for growth in the presence of *p*-coumaric acid, while the growth of the LPD1 mutant at acidic pHs is strongly inhibited by *p*-coumaric acid. These results are consistent with those obtained recently for *E. coli* growth with inhibitory concentrations of ferulic or vanillic acid (45). Therefore, PDC synthesis in *L. plantarum* appears to be the most efficient cellular response to quickly convert *p*-coumaric acid into a less toxic derivative. The low PDC2 and PAR activities are much less efficient as detoxification systems, and their biological significance remains to be established. Two mechanisms are known to be involved in phenolic acid toxicity under acidic growth conditions: the dissipation of the Δ pH and a specific toxicity of phenolic acids. At pHs lower than their pK_a (about 4.5), phenolic acids are essentially found in the protonated form. They

FIG. 7. Proposed pathway for the degradation of *p*-coumaric acid in *L. plantarum*. The arrow thickness represents the relative intensity of enzymatic activity. PDC, *p*-coumaric acid decarboxylase; PDC2, phenolic acid decarboxylase; PAR, phenolic acid reductase; VPR, putative 4-vinyl phenol reductase; DEC, putative phloretic acid decarboxylase.

can enter the bacterial cell by diffusion, while the released H⁺ protons depress the internal pH, thereby inhibiting bacterial growth. Recent studies have shown that in *S. cerevisiae*, the H⁺-ATPase which pumps protons out of the cell is induced by cinnamic acid at inhibitory concentrations (10). This proton pump could be activated in order to counteract dissipation of the transmembrane proton motive force and to restore the ΔpH (5). Such a response is probably the primary mechanism to overcome phenolic acid toxicity, since *S. cerevisiae* displays a very weak PAD activity (13), 5,000-fold weaker than the *L. plantarum* PDC. Similarly, the corresponding H⁺-ATPase of *L. plantarum* (40) could counteract the dissipation of ΔpH upon the entrance of phenolic acids. Proton pump activation uses ATP molecules, which are no longer available for biosynthesis purposes, explaining the lower growth rate and lower final biomass in medium supplemented with *p*-coumaric acid. The specific molecular toxicity of *p*-coumaric acid, which is evident in the LPD1 mutant, is considerably reduced by the high PDC activity in the wild-type strain, LPNC8.

In conclusion, knockout of the *pdc* gene from *L. plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. However, these activities are about 100-fold lower than that of PDC. Therefore, PDC activity appears to be one of the major components of the stress response caused by phenolic acids, particularly in acidic media, which are the natural habitats of lactic acid bacteria. Our mutant provides a convenient model for studying mechanisms of the stress response which involve specific phenolic acid-dependent regulation systems. Such regulation systems are currently under investigation in our laboratory.

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