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Molecular characterization of the phenolic acid metabolism in the lactic acid bacteria *Lactobacillus plantarum*

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Abstract — The lactic acid bacteria *Lactobacillus plantarum* displays substrate-inducible decarboxylase activities on *p*-coumaric, caffeic and ferulic acids. Purification of the *p*-coumaric acid decarboxylase (PDC) was performed. Sequence of the N-terminal part of the PDC led to the cloning of the corresponding *pdc* gene. Expression of this gene in *Escherichia coli* revealed that PDC displayed a weak activity on ferulic acid, detectable in vitro in the presence of ammonium sulfate. Transcriptional studies of this gene in *L. plantarum* demonstrated that the *pdc* transcription is phenolic acid-dependent. A mutant deficient in the PDC activity, designated LPD1, was constructed to study phenolic acid alternate pathways in *L. plantarum*. LPD1 mutant strain remained able to metabolize weakly *p*-coumaric and ferulic acids into vinyl derivatives or into substituted phenyl propionic acids. These results indicate that *L. plantarum* has a second acid phenol decarboxylase enzyme and also displays inducible acid phenol reductase activity. Finally, PDC activity was shown to confer a selective advantage for LPNC8 grown in acidic media supplemented with *p*-coumaric acid, compared to the LPD1 mutant devoid of PDC activity.

phenolic acid / phenolic acid decarboxylase / phenolic acid reductase / *Lactobacillus plantarum*

Résumé — Caractérisation moléculaire du métabolisme des acides phénol chez *Lactobacillus plantarum*. La bactérie lactique *Lactobacillus plantarum* est capable de décarboxyler les acides *p*-coumarique, férulique et caféique. La purification de l'acide *p*-coumarique décarboxylase (PDC) a été réalisée. La détermination de la séquence N-terminale de cette enzyme a permis de cloner le gène *pdc* correspondant. L'expression de ce gène chez *Escherichia coli* révèle que la PDC possède une faible activité sur l'acide férulique, détectable in vitro uniquement en présence de sulfate d'ammonium. L'étude des ARN messagers du gène *pdc* chez *L. plantarum* montre que la transcription de ce gène dépend des acides phénols. La construction d'un mutant déficient pour l'activité PDC, nommé LPD1 a été réalisée afin d'étudier les métabolismes secondaires des acides phénols chez *L. plantarum*. Ce mutant LPD1 reste capable de dégrader les acides phénols en dérivés de type vinyl phénol ou en acides phényl propioniques. Ces résultats indiquent que *L. plantarum* possède une seconde acide

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phénol décarboxylase ainsi qu'une activité acide phénol réductase. Enfin, l'activité PDC confère à *L. plantarum* un avantage sélectif pour sa croissance en milieu contenant des acides phénols, comparé à la souche LPD1 dépourvue d'activité PDC.

acide phénol / acide phénol décarboxylase / acide phénol réductase / *Lactobacillus plantarum*

1. INTRODUCTION

Phenolic acids, of which ferulic and *p*-coumaric acids are the main constituents, represent 1–5% (m/w dry weight) of tropical grasses, of maize and wheat bran and of sugarbeet pulp [22]. These acids are covalently bound to polysaccharides (xylan and lignin) in plant cell walls to ensure cell wall rigidity [12]. A wide range of microorganisms display cinnamoyl esterase activities which have been shown to release phenolic acids from plant cell wall polymers [9]. Under their free form, phenolic acids are considered differently according to the microorganisms. Phenolic acids have inhibitory effect on the growth of most microorganisms [4, 34]. It is the reason why they are used as antimicrobial agents in the food industry [25]. Phenolic acids also serve as a signal and induce virulence gene expression in the plant-associated *Agrobacterium tumefaciens* [20]. Some *Pseudomonas* strains as well as *Acinetobacter calcoaceticus* are able to use these acids as the sole source of carbon for growth [17, 24, 28, 30]. Moreover, ferulic acid is exploited to produce value-added aromatic compounds, such as vanillin. This bioconversion is performed by two fungi, *Aspergillus niger* and *Pycnoporus cinnabarinus* [21].

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 into 4-ethyl derivatives. Phenolic acid decarboxylases (PAD) have been characterized in the yeasts *Saccharomyces cerevisiae* and *Brettanomyces annomalus* [10, 15] and also in the bacteria *Bacillus pumilus* [33], *Lactobacillus plantarum* [6] and *Bacillus subtilis* [8]. 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 [31]. Some of these volatile phenols, particularly vinyl and ethyl guaiacol (generated from ferulic acid) are useful aromatic chemicals [19] or contribute naturally to aroma in wine [16] and other fermented foods and beverages. On the contrary, some volatile phenols, such as vinyl and ethyl phenol (from *p*-coumaric acid) are most often considered phenolic off-flavors and are responsible for alterations in organoleptic properties [15].

The interest in improving our understanding of phenolic acid biodegradation is multiple. Firstly, as has been shown for *S. cerevisiae*, PAD activity may confer a selective advantage upon microorganisms during growth on plant, where PAD expression could constitute a stress response induced by phenolic acid [10, 18]. Secondly, phenol derivatives are valuable intermediates in the biotechnological production of new flavor and flagrance chemicals [19]. Thirdly, they are regarded as a source of phenolic off-flavors in many beers and wines, due to their characteristic aroma and their low threshold detection [16, 29].

Several strains of lactic acid bacteria isolated from wine were tested for their activities to metabolize ferulic and *p*-coumaric acids [5]. It was shown that ferulic and *p*-coumaric acids were strongly decarboxylated by growing cultures of *L. plantarum* and *Pediococcus pentosaceus.* The decarboxylase activity was only detected for

bacteria grown with these substrates suggesting that this activity was inducible. Moreover, growth of *L. plantarum* and *P. pentosaceus* strains was not particularly affected by presence of ferulic and *p*-coumaric acids in growth medium, indicating that decarboxylation of these compounds may confer resistance to inhibitory effects of phenolic acids. The ubiquitous lactic acid bacteria, *L. plantarum*, used as a malolactic starter in wine and as a lactic starter for many vegetable fermentations, was selected as a model for studying phenolic acid metabolism. This bacterium displayed substrate-inducible decarboxylase activity on *p*-coumaric, ferulic, and caffeic acids. Purification of the *L. plantarum* *p*-coumaric acid decarboxylase (PDC) enables the biochemical characterization of this enzyme, and the cloning of the corresponding *pdc* gene. A food grade mutant strain, deficient in PDC activity, was constructed to modify the aromatic property of the strain, to study the metabolic pathway of phenolic acids, and the influence of PDC activity on the growth in the presence of phenolic acids.

2. MATERIALS AND METHODS

2.1. Microorganisms and culture conditions

The *L. plantarum* LPNC8 and LPD1 were grown at 37 °C in MRS medium without shaking [11]. *E. coli* TG1 was grown at 37 °C on LB medium under shaking conditions [3]. Antibiotics were used in the following concentrations: erythromycin 100 mg·L^{-1} for *E. coli* and 5 mg·L^{-1} for *L. plantarum*.

2.2. DNA manipulation and transformation procedures

Standard procedures described by Sambrook et al. [26] were used for DNA manipulation. *L. plantarum* chromosomal DNA was prepared using the method described by Posno et al. [23]. PCR products were purified with the Jet Pur Kit (Genomed, Bioprobe, Montreuil, France) and sequenced by the dideoxy chain termination method [27] with the Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science Inc., Cleveland, Ohio, USA) in accordance with the recommendations of the manufacturer. PCR amplification was performed using 0.1 μg DNA template with 0.5 U of *Taq* DNA polymerase (Appligene) under standard conditions, in an automatic Hybaid DNA thermocycler (Hybaid Ltd., Teddington, United Kingdom). *E. coli* and *L. plantarum* strains were transformed by electroporation as described by Dower et al. [14] and Aukrust and Nes [1], respectively.

2.3. Preparation of whole cell suspensions, cell-free extracts and assay of phenolic acid degradation

Cells of *L. plantarum* grown in MRS medium and *E. coli* grown in LB medium were harvested by centrifugation, washed with 25 mmol·L^{-1} potassium phosphate buffer (pH 6.0) and resuspended in the same buffer at a final concentration of 5 g (dry weight) of cells per liter. Reactions were started by adding *p*-coumaric or ferulic acids. During incubation, samples were centrifuged and supernatants were diluted 50-fold in Stop buffer (20 mmol·L^{-1} Tris-HCl, 3 g·L^{-1} SDS to stop activity, pH 6) prior to analysis. For cell-free extract preparation, cells were harvested as described above and disrupted with a French press at 1.2×10^8 Pa. Kinetic reactions were started by addition of the substrate and samples were diluted in Stop buffer. Phenolic acid consumption and derivative production was monitored by UV-spectrophotometry scanning (from 180 to 330 nm using quartz cuvettes in a Beckman DU600 spectrophotometer) as previously described [2, 5, 13].

3. RESULTS

3.1. Phenolic acid metabolism in *L. plantarum*, purification and characterization of the *p*-coumaric acid decarboxylase

L. plantarum is a lactic acid bacteria which is able to decarboxylate *p*-coumaric and ferulic acids into 4-vinyl phenol and 4-vinyl gaiacol respectively; however, we did not know if this bacteria had one or two different phenolic acid decarboxylases (PADs). Previous experiments revealed that *L. plantarum* induced with 3 mmol·L^{-1} *p*-coumaric acid displayed decarboxylase activity on *p*-coumaric acid (0.6 µmol·min^{-1}·mg^{-1}). While *L. plantarum* induced with 3 mmol·L^{-1} ferulic acid displayed a similar activity on *p*-coumaric acid and also a weak activity (0.01 µmol·min^{-1}·mg^{-1}) on ferulic acid [7]. These results indicate that two distinct PADs are present in *L. plantarum*.

The enzyme induced by *p*-coumaric acid, which metabolized *p*-coumaric acid, was purified and designated *p*-coumaric acid decarboxylase (PDC) [7]. SDS-page analysis of this purified PDC revealed the presence of single band of 23.5 kg·mol^{-1}. Determination of PDC molecular mass indicated that this enzyme of 93 kg·mol^{-1} was a homotetramer. Full enzyme activity was obtained in 25 mmol·L^{-1} phosphate buffer with an optimal pH of 6.0 and temperature of 30 °C. Only *p*-coumaric and caffeic acids were decarboxylated by the PDC in the conditions tested in vitro [7]. This result indicated that *L. plantarum* should produce a second PAD, specific for ferulic acid and induced by this substrate. The N-terminal amino-acid sequence of the purified PDC was obtained.

3.2. Cloning of the *pdc* gene, transcriptional analysis and characterization of the recombinant PDC in *Escherichia coli*

By using degenerate primers designated from the first 19 N-terminal amino-acid sequence of PDC, a 57-bp fragment was amplified from *L. plantarum* by PCR, was cloned in *E. coli* vector and was sequenced [6]. Its deduced amino-acid sequence proved to be identical to the 19 amino-acids of the PDC enzyme. This nucleotide sequence was used as a probe to screen the *L. plantarum* genomic library. Only one clone, designated pJPDC1 with a 2.3-kb insertion, hybridized with this probe. An open reading frame (ORF), with a coding capacity of 522-bp was detected and encoding for a 174 amino-acid deducted product, which N-terminal amino acid sequence was identical to the N-terminal extremity of the purified PDC [6]. The primary structure of the deduced polypeptide displays 64% identity with the FDC from *Bacillus pumilus* [33]. This 522-bp ORF was identified as the *L. plantarum pdc* gene. A DNA probe encompassing about 300 nucleotides from the *pdc* gene of *L. plantarum* was used to screen a *B. subtilis* and a *P. pentosaceus* genomic library and led to the cloning of the genes *pad* and *padA* of, respectively, *B. subtilis* [8] and *P. pentosaceus* (Barthelmebs L., Lecomte B., Diviès C., Cavin J.-F., submitted for publication).

Although these four PADs exhibited 64% of amino-acid sequence identity, the purified enzymes had different characteristics. First, PDC was a homotetramer while FDC and BSPAD were homodimers. These two purified enzymes displayed about the same high activity (500 nmol·min^{-1}·mg^{-1}) for *p*-coumaric and ferulic acids, contrary to the PDC and the PPPAD which displayed no activity on ferulic acid. Moreover, the sequence alignment of the four enzymes showed that the main differences between these three proteins are located in the N- and C-terminal parts (Fig. 1). Nevertheless, no homology was found between these PADs with the other decarboxylases, including the phenylacrylic acid decarboxylase (PAD1) cloned from *Saccharomyces cerevisiae*, which was able to decarboxylate, with a weak activity, *p*-coumaric and ferulic acids.

Figure 1. Comparison between the sequence of the *B. subtilis* PAD (PAD-BS), the *B. pumilus* FDC (FDC-BP), the *L. plantarum* PDC (PDC-LP) and the *P. pentosaceus* PAD (PAD-PP). The sequences were aligned using the Clustal program. Identical residues are boxed and shaded. The numbers on the right correspond to the amino-acid position in the protein sequence.

Transcriptional analysis of the *pdc* gene was carried out with RNA from *L. plantarum* grown with or without 1.2 mmol·L^{-1} *p*-coumaric acid in the medium. A *pdc* transcript of 700 nucleotides was detected in the induced RNA while no transcript was detected in the uninduced RNA, even by Northern blotting or by primer extension experiments. These results indicate that *pdc* transcription is acid phenol-dependent. The same results were obtained for the transcription of the *pad* from *B. subtilis* [8] and the *padA* from *P. pentosaceus* (Barthelmebs L., Lecomte B., Diviès C., Cavin J.-F., submitted for publication).

P-coumaric and ferulic acid metabolism was tested in whole cells and corresponding cell-free extract of recombinant *E. coli* TG1 (pJPDC1) strain grown with or without *p*-coumaric acid as inducer. No PDC activity was found in the control *E. coli* TG1 (pJDC9). Induced and uninduced whole cells and cell-free extracts displayed similar decarboxylase activity of 5 to 12 µmol·min^{-1}·mg^{-1} on *p*-coumaric acid. In induced and uninduced whole cells, ferulic acid was decarboxylated at a rate of about 40 nmol·min^{-1}·mg^{-1}, while the corresponding cell-free extract displayed no detectable activity on ferulic acid in phosphate buffer. Reaction conditions were then modified by independently varying pH and temperature, and by adding glycerol or salts to kinetic buffer. Ferulic acid decarboxylase activity was stimulated in cell-free 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.

3.3. Phenotypic analysis of the *L. plantarum* strain deficient in PDC activity

In order to investigate alternate pathways for phenolic acid degradation, a *L. plantarum* strain deficient in PDC activity was constructed [2]. The procedure used led to a completely stable chromosomal deletion within the *pdc* gene through a two-step homologous recombination process. In short, a copy (A-C) of the *pdc* gene lacking 200-bp internal region (B) (Fig. 2), was cloned on a vector. A frame shift in the deleted copy created a stop codon, which caused the synthesis of a truncated polypeptide of 51 amino acids. Contrary to the *E. coli* TG1 (pJPDC1) clone, no PDC activity was detected on either substrate in whole cells or cell-free extracts of the *E. coli* TG1 strain bearing this plasmid. This plasmid, which is suitable for generating the *L. plantarum* mutant, was introduced in *L. plantarum* by electroporation. Campbell-type integration of the vector in the chromosome (involving the A or C region) resulted in a wild-type phenotype. Secondary excision through intrachromosomal recombination within the other region of homology (C or A) led to a deletion of the internal B region of the *pdc* gene. The mutant strain was named LPD1. Southern blotting and DNA sequencing were performed on the LPD1 DNA to confirm the deletion [2].

In order to characterize the phenolic acid metabolism of LPD1 mutant, growing

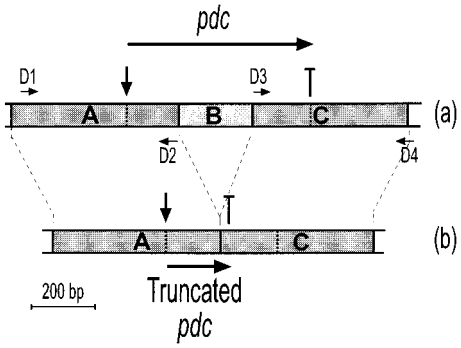

Figure 2. Physical map of the *pdc* locus in: (**a**) the wild-type strain *L. plantarum* and (**b**) the mutant strain LPD1. Horizontal arrows represent the two ORFs and their orientation. The start sites are indicated by a vertical arrow and the stop codons by a T. The position and orientation of the primers are indicated by short horizontal arrows.

cultures of LPD1 were divided into 5 samples and induced with 1.2 or 3 mmol·L^{-1} *p*-coumaric acid and ferulic acid or non induced. Whole resting cell suspensions were prepared in 25 mmol·L^{-1} phosphate buffer (pH 6.0) and tested for *p*-coumaric and ferulic acid metabolism. No acid degradation was detected in the uninduced whole cells. Cells induced with ferulic acid (1.2 and 3 mmol·L^{-1}) displayed a low decarboxylase activity of about 12 nmol·min^{-1}·mg^{-1} on *p*-coumaric and ferulic acids, indicating that *L. plantarum* possesses a second PDC. This activity was not detected in cells induced with 1.2 mmol·L^{-1} *p*-coumaric acid, although the two substrates were degraded into a product which could be either substituted phenyl propionic acids or 4-ethyl derivatives. Production of 4-ethyl derivatives from phenolic acids requires the prior formation of 4-vinyl derivatives or substituted phenyl propionic acids. As 4-vinyl derivatives were never detected in kinetic experiments, phenolic acids were likely reduced into substituted phenyl propionic acids, and subsequently decarboxylated into 4-ethyl derivatives. Cells induced with 3 mmol·L^{-1} *p*-coumaric seem to display the two activities of decarboxylase and reductase. Since a reduced cofactor is generally required for enzymatic reduction, 20 mmol·L^{-1} glucose was added to whole cells prior to starting the kinetics, in order to stimulate glycolysis and increase the pool of reduced cofactors. This addition stimulated a reductase activity of 10 nmol·min^{-1}·mg^{-1} on the two phenolic acids for whole cells induced by *p*-coumaric acid (1.2 or 3 mmol·L^{-1}) and by 1.2 mmol·L^{-1} ferulic acid. Decarboxylase activity was nevertheless detected in whole cells induced by 3 mmol·L^{-1} ferulic acid. Taken together, our results indicate the presence, in *L. plantarum*, of a second phenolic acid decarboxylase (named PDC2), greatly 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.

3.4. Influence of *p*-coumaric and ferulic acid concentration on the growth of wild type and LPD1 mutant strains at different pH

Increasing concentrations of *p*-coumaric and ferulic acids (0, 1.2, 3, and 6 mmol·L^{-1}) were tested for their effect on growth of the wild-type and LPD1 mutant strains in MRS broth at pH 6.5. For the wild-type strain, addition of 1.2 or 3 mmol·L^{-1} *p*-coumaric acid and 1.2 mmol·L^{-1} ferulic acid in the medium had no apparent effect on growth (Figs. 1a, 2a and 3). Addition of 6 mmol·L^{-1} *p*-coumaric acid and 3 or 6 mmol·L^{-1} ferulic acid increased the lag phase, but the final biomass reached a level similar to that of the control culture without *p*-coumaric or ferulic acids. The growth of LPD1 mutant strain was not different to that of the wild-type strain in presence of increasing concentrations of ferulic acid (Figs. 2b and 3). LPD1 growth was weakly reduced with 1.2 mmol·L^{-1} *p*-coumaric acid but was significantly affected with 3 or 6 mmol·L^{-1} *p*-coumaric acid in the medium, with a final biomass reaching only 60 and 80% compared to that of the control without acid (Figs. 1b and 3).These results seem to indicate that *p*-coumaric was more toxic than ferulic acid for *L. plantarum* cells devoid of their major decarboxylase activity. In order to investigate the influence of acid uptake on growth rate, cultures were performed at pH 4.5 in MRS broth with the three *p*-coumaric and ferulic acid concentrations previously used (Fig. 4). At pH 4.5, the growth of the wild-type strain remained unaffected in the presence of 1.2 and 3 mmol·L^{-1} *p*-coumaric and 1.2 mmol·L^{-1} ferulic acids, was partially reduced with 3 mmol·L^{-1} *p*-coumaric and 1.2 mmol·L^{-1} ferulic acids and was totally inhibited with 6 mmol·L^{-1} ferulic acid (Figs. 1a, 2a and 4). On the other hand, LPD1 growth was strongly inhibited in the presence of 1.2 and 3 mmol·L^{-1} *p*-coumaric acid and totally inhibited with 6 mmol·L^{-1} *p*-coumaric acid

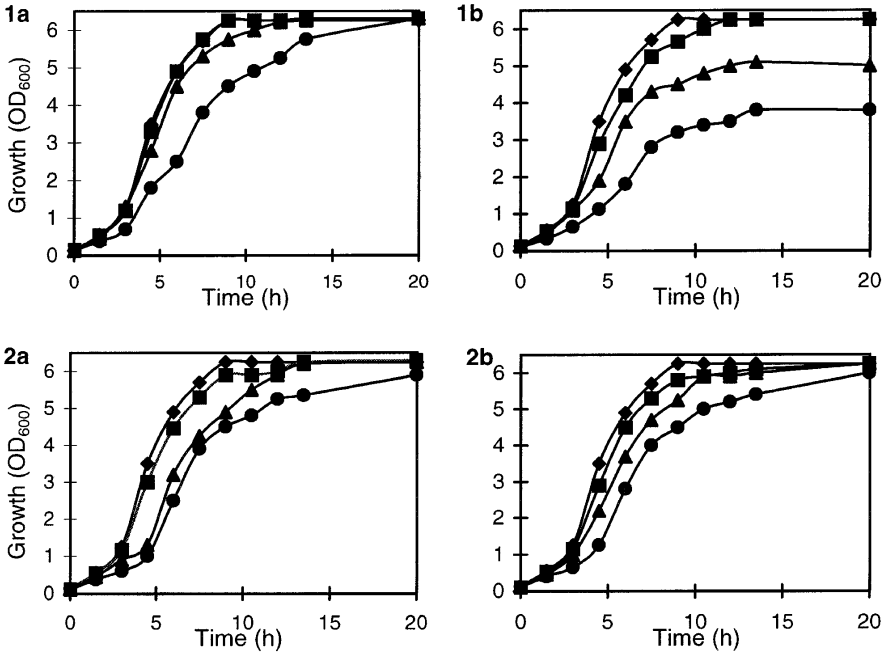

Figure 3. Growth of *L. plantarum* wild-type (a) and LPD1 (b) strains at (1) different *p*-coumaric acid concentrations (in mmol·L^{-1}), ◆: 0; ■: 1.2; ▲: 3; ●: 6 and at (2) different ferulic acid concentrations (in mmol·L^{-1}), ◆: 0; ■: 1.2; ▲: 3; ●: 6, at pH 6.5.

(Figs. 1b and 4). LPD1 growth was similar to *L. plantarum* wild-type growth with the same concentrations of ferulic acid (Figs. 2b and 4). Taken together, these results indicated that the toxicity of *p*-coumaric and ferulic acids is enhanced under low initial pH condition. Nevertheless, PDC activity seems to confer resistance to *p*-coumaric acid toxicity.

4. DISCUSSION

Study of phenolic acid metabolism in *L. plantarum* wild-type and mutant strain deficient in PDC activity, revealed that this lactic acid bacteria possesses at least three substrate inducible enzymatic activities able to metabolize phenolic acids. PDC enzyme displays the higher activity on *p*-coumaric acid, and a 50-fold lower activity on ferulic acid, detectable in vitro only when ammonium sulfate or NaCl were added in the reaction buffer. *L. plantarum* is, to our knowledge, the first microorganism which displays two distinct and functional PAD activities. In *B. subtilis*, a *yclB* gene which deduced polypeptide displayed 35% amino acid sequence identity with the PAD1 from *S. cerevisiae* which was identified by genome sequencing [10, 32]. Nevertheless, the functionality of the YclB protein was not proved. The analysis of the LPD1 mutant strain also revealed that *L. plantarum* converts phenolic acids into substituted phenyl propionic acids, therefore indicating that *L. plantarum* appears to have a weak *p*-coumaric and ferulic acid reductase activity, induced by both substrates and mostly active when glucose is added. This pathway

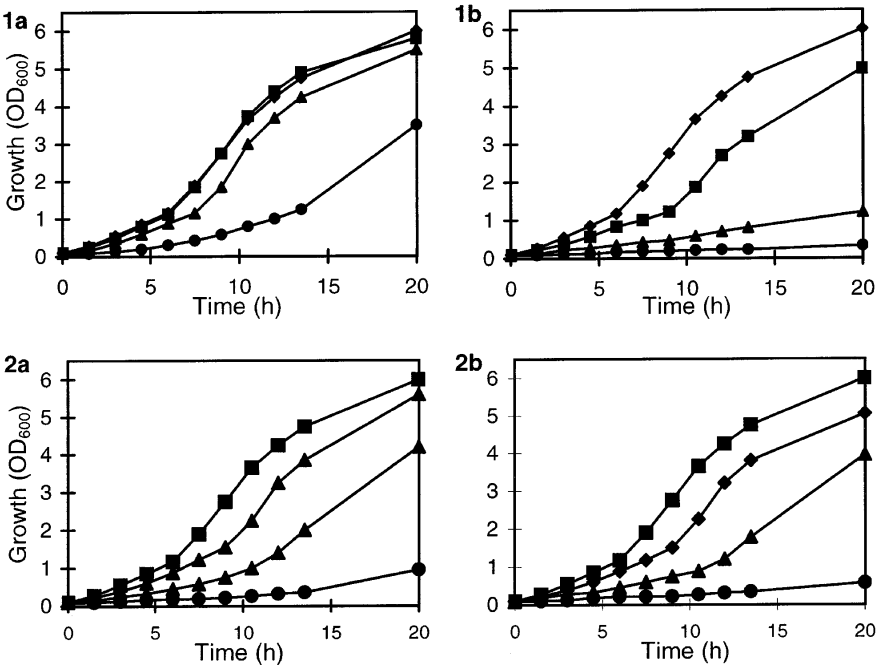

Figure 4. Growth of *L. plantarum* wild-type (**a**) and LPD1 (**b**) strains at (1) different *p*-coumaric acid concentrations (in mmol·L^{-1}), ◆: 0; ■: 1.2; ▲: 3; ●: 6, and at (2) different ferulic acid concentrations (in mmol·L^{-1}), ◆: 0; ■: 1.2; ▲: 3; ●: 6, at pH 4.5.

has been previously shown in *Lactobacillus pastorianus* [31] and also in cells of *S. cerevisiae*, grown under argon condition [19].

We have also demonstrated that functional PDC enzyme clearly confers a selective advantage on the wild-type strain *L. plantarum* for growing in the presence of *p*-coumaric acid, while growth of the LPD1 mutant at acidic pH is strongly inhibited by *p*-coumaric acid. 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. Mechanism of induction of the PDC synthesis is currently in progress. The absence of detectable PDC activity with the absence of detectable corresponding mRNA in the uninduced cells indicate that the *pdc* transcription is phenolic acid-dependant, making this regulatory system a potential tool for gene expression studies in lactic acid bacteria or other gram-positive bacteria.

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