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Characterization of a Nitroreductase with Selective Nitroreduction Properties in the Food and Intestinal Lactic Acid Bacterium *Lactobacillus plantarum* WCFS1

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Nitroreductases reduce nitroaromatic compounds and other oxidants in living organisms, having interesting implications in environmental and human health. A putative nitrobenzoate reductase encoding gene (lp_0050) was recently annotated in the completed DNA sequence of lactic acid bacterium *Lactobacillus plantarum* WCFS1 strain. In this research, this *L. plantarum* gene was cloned and expressed, and the corresponding protein (PnbA) was biochemically characterized. This *L. plantarum* PnbA reductase is a 216 amino acid residue FMN-flavoprotein, which exhibits 23% identity with *Pseudomonas putida* and *Ralstonia eutropha* nitroreductases and <11% identity with those from enterobacteria such as *E. cloacae*. This reductase also showed 32−43% identity (65−72% similarity) to predicted PnbA proteins from other lactic acid bacteria. It utilized a wide range of electron acceptors including dichlorophenolindophenol (DCPIP), nitroblue tetrazolium (NBT), ferricyanide, and quinones (menadione, benzoquinone), but not pyridinium cations (paraquat and *N*-methyl-β-carbolines), and it was inhibited by dicoumarol and diphenyliodonium. HPLC-MS and spectroscopic data showed that it specifically catalyzed the reduction of the 4-nitroaromatic group to the corresponding hydroxylamine in the presence of NAD(P)H. Kinetics parameters (V_{max} and K_m) showed a higher efficiency for the reduction of 2,4-dinitrobenzoate than for the reduction of 4-nitrobenzoate. It was chemoselective for the reduction of 4-nitrobenzoates, being unable to reduce other nitroaromatics. Then, *L. plantarum* PnbA reductase might be more specific than other microbial nitroreductases that reduce a wider range of nitroaromatic compounds. The physiological and functional role of nitroreductases remain unknown; however, their presence in lactic acid bacteria widely occurring in foods and the human intestinal tract should be of further interest.

KEYWORDS: Nitroreductase; lactic acid bacteria; *Lactobacillus plantarum*; nitrobenzoate; nitrocompounds; quinone reductase; pyridinium cations; *N*-methyl-β-carbolines

INTRODUCTION

Lactic acid bacteria are common in foods and the mammalian gut. Among them, *Lactobacillus plantarum* is a versatile lactic acid bacterium encountered in a range of environmental niches that has a proven ability to survive and colonize the intestinal tract of humans and other mammals (*1*, *2*). The complete genome sequence of *L. plantarum* WCFS1 has become recently available (*3*), making this bacterium a suitable model to explore the molecular mechanisms underlying the targeted intestinal properties of this species (*1*, *4*). From its complete genome sequence analysis, a sequence (lp_0050) putatively encoding a protein annotated as *p*-nitrobenzoate reductase (PnbA) was found, although it has not been further produced and/or characterized. Nitroreductases usually catalyze the reduction of nitrocompounds that are cytotoxic and mutagenic (*5*, *6*). Despite their toxicity, some bacteria have been found to harbor enzymes for the metabolism of nitroaromatics, and they have been considered to be of great potential application in environmental bioremediation and biocatalysis and as chemotherapeutic agents following the bioactivation of prodrugs (*7*−*14*).

Nitroreductases belong to two main classes: oxygen-insensitive (type I) and oxygen-sensitive (type II) (*15*). The former abounds in bacteria and fungi (*16*) and the latter in mammalian systems. Oxygen-sensitive nitroreductases usually catalyze one-electron reduction, yielding a nitro anion radical (*17*) that reacts with oxygen to form a superoxide radical regenerating the nitro group. In this "futile cycle" pyridine nucleotides are oxidized without net reduction of the nitro group. In the absence of oxygen further reduction of the nitroaromatic may yield nitroso, hydroxylamine, and amino derivatives (*17*). Oxygen-insensitive nitroreductases are a family of bacterial FMN-containing enzymes that catalyze the reduction of nitrocompounds, producing nitroso, hydroxylamine, and/or amino derivatives (*7*, *11*, *18*). These nitroreductases have been studied in a number of bacteria including enterobacteria (e.g. *Escherichia coli*, *Salmonella typhimurium*, *E. cloacae*),

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Pseudomonas, and *Rhodobacter capsulatus* (*19*−*26*) and exhibit varying substrate specificities, being able to reduce a wide range of nitroaromatics such as nitrophenols, nitrobenzenes, and nitrobenzoates. So far, the real physiological and functional role of microbial nitroreductases remains unknown (*7*), but it may include a possible role in quinone (or oxidants) reduction and detoxification (*23*), oxidative stress (*27*, *28*), bioluminescence (*29*), cobalamin synthesis (*30*), or even a function involving specific degradation pathways of nitrocompounds (*7*, *13*, *31*).

A large number of nitroaromatics are present in the environment because of their use in manufacturing processes, as antimicrobial agents or generated as byproducts of combustion processes. They could be biodegraded by using selected microorganisms and/or nitroreductases (*7*, *13*, *32*). Nitroaromatic compounds have also attracted considerable health concern because their metabolism through reductive pathways may lead to potent genotoxic and/or mutagenic metabolites (*5*, *33*, *34*). Indeed, nitrocompounds are able to generate reactive nitrogen oxide species, which readily react with biological molecules (*35*). Although the metabolic pathways of these compounds are complex, nitroreductases appear to have a central role in their bioactivation (*16*, *36*). Thus, enzymatic reduction by nitroreductases gives rise to reactive intermediates that can undergo nucleophilic additions with DNA and other macromolecules, suggesting a possible mechanism for their cytotoxicity (*6*).

Lactic acid bacteria from foods and gut might metabolize nitroaromatics having possible environmental, health, and toxicological implications (*1*, *26*). In this regard, the goal of this work was to study the biochemical and functional characteristics of a novel Pnb reductase from *L. plantarum* WCFS1. The gene encoding the PnbA reductase from this bacterium was cloned and the recombinant protein overexpressed in *Escherichia coli*. This functional nitroreductase was a FMN flavoenzyme able to reduce several electron acceptors, including various quinones but not pyridinium cations, and it was selective for the reduction of specific 4-nitroaromatic compounds, exhibiting a potent reduction for 4-nitrobenzoate and 2,4-dinitrobenzoate to 4-hydroxylamine metabolites. Then, this nitroreductase present in lactic acid bacteria might play a role in the metabolism and reduction of aromatic compounds.

MATERIALS AND METHODS

Chemicals and Reagents. FMN, NADPH, NADH, dicoumarol, diphenyliodonium chloride (DPI), dichlorophenolindophenol (DCPIP), ferricyanide, nitroblue tetrazolium (NBT), cytochrome C, menadione (2-methyl-1,4-naphthoquinone), 1,4-benzoquinone, *p*-nitrophenyl-β(D)-glucopyranoside, 1,1′-dimethyl-4,4′-bipyridinium dichloride hydrate, 4-nitrobenzoic acid, 3-nitrobenzoic acid, 2-nitrobenzoic acid, 2,4-dinitrophenol, 5-nitroindazole, and 2-amino-4-nitrophenol were obtained from Sigma-Aldrich, 2,4-dinitrobenzoic acid and nitrobenzene were from Fluka, 3,5-dinitrosalicilic acid was from Merck, and picric acid was from Probus. 2-methyl-β-carbolinium iodides (2-methyl-9*H*-pyrido[3,4-*b*]indole) (i.e., 2-methylnorharman and 2-methylharman cations) were synthesized from norharman and harman with methyl iodide in acetone (*37*, *38*). Several nitroderivatives such as *N*-(2-nitrophenyl)-L-proline, *N*-(2-nitrophenyl)pipecolic acid, *N*-(2,4-dinitrophenyl)glycine, and *N*-(2-nitrophenyl)glycine were synthesized as previously described (*39*). *p*-Hydroxylaminobenzoic acid was synthesized from 4-nitrobenzoic acid by reduction with Zn in the presence of NH_4Cl (*40*). For that, 17 g of 4-nitrobenzoic acid was dissolved in water and sodium hydroxide. After the addition of 20 g of ammonium chloride, the mixture was cooled and 15 g of zinc dust was added gradually with stirring. After 0.5 h of stirring, the zinc sludge was filtered off. Hydrochloric acid was slowly added to the filtrate, and amorphous flakes, which separated while the reaction was still neutral, were removed by filtration. Upon the addition of more hydrochloric acid to the filtrate, white needles of *p*-hydroxylaminobenzoic acid separated and were characterized by spectral data. This compound was used for quantification using the corresponding calibration curves.

Bacterial Strains and Plasmids. The *L. plantarum* strain that has been completely sequenced, *L. plantarum* WCFS1 (NCIMB 8826), was used in this study. *E. coli* DH5α was used for all DNA manipulations. *E. coli* JM109 (DE3) was used for expression in pURI3 vector (*41*, *42*). The *L. plantarum* strain was grown in MRS medium at 30 °C without shaking. *E. coli* strains were cultured in Luria−Bertani (LB) medium at 37 °C and 200 rpm. When required, ampicillin or chloramphenicol was added to the medium at a concentration of 100 or 34 μg/mL, respectively. Chromosomal DNA, plasmid purification, and transformation of *E. coli* were carried out as described elsewhere (*43*).

Expression and Purification of *L. plantarum* PnbA. The gene coding for the putative *p*-nitrobenzoate reductase, *pnbA* (lp_0050) from *L. plantarum* WCFS1, was cloned and overexpressed in pURI3 vector to avoid the enzyme and ligation steps during the cloning (*41*). Expression vector pURI3 was constructed on the basis of the commercial expression vector pT7-7 (USB) but containing a leader sequence with a six-histidine affinity tag. The *pnbA* gene was PCR amplified with Hot-start Turbo *Pfu* DNA polymerase by using the primers 321 (5′-*CATCATGGTGACGATGACGATAAG*atggaaacaattaaagcgattcaca) and 322 (5′-*AAGCTTAGTTAGCTATTATGCG*ttagttgataatatgcaaaacttgcgg) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *pnbA* gene sequence are written in lowercase letters). The 648 pb purified PCR product was inserted into the pURI3 vector by using a restriction enzyme- and ligation-free cloning strategy described previously (*41*). *E. coli* DH5α cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* JM109(DE3) (pLysS) cells for expression.

Cells carrying the recombinant plasmid, pURI3-pnbA, were grown at 37 °C in LB media containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL), until they reached an optical density at 600 nm of 0.4, and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 °C for 20 h and collected by centrifugation. Cells were resuspended in 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl. Crude extracts were prepared by French press lysis of the cell suspension (three times at 1100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47000*g* for 30 min at 4 °C.

The supernatant was filtered through a 0.45 μm filter and applied to a His-Trap-FF crude chelating affinity column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl containing imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl containing 10 mM imidazole to the same buffer but containing 500 mM imidazole. Fractions containing the His_6-tagged protein were pooled and dialyzed overnight at 4 °C against 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl in a membrane (3500 cuttoff). The purity of the enzyme was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) in Tris−glycine buffer. Protein concentration was measured according to the method of Bradford using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Reductase Assays with Electron Acceptors. The activity of PnbA reductase was monitored using several electron acceptors as substrates as follows: (a) Reduction of dichlorophenolindophenol (DCPIP) was assayed in a reaction mixture containing 100 mM phosphate buffer, pH 7.4, 500 μM NADPH, 200 μM DCPIP, and PnbA reductase (1−5 μg/mL protein) in a final volume of 0.5 mL. The reaction was initiated by the addition of substrate, and the rate of DCPIP reduction with time was determined (λ = 600 nm; ε = 21.5 mM^{-1} cm^{-1}). Reaction rate was also calculated in the absence of enzyme or NADPH as controls and in the presence of diphenyliodonium (0−10 mM) or dicoumarol (0−500 μM) used as inhibitor. Reduction of DCPIP was also used to determine the activity in the presence of NADH (500 μM) by replacing NADPH as electron donor and to study the optimal temperature of reduction after the enzyme had been incubated for 20 min at different temperatures. (b) Reduction of cytochrome C in the presence or absence of menadione as an electron acceptor was measured using a reaction mixture containing 90 mM Tris-HCl buffer, pH 7.4, 500 μM NADPH, menadione (0 or 20 μM), 0.96 mg/mL of cytochrome C, and PnbA reductase (1−5 μg/mL protein)

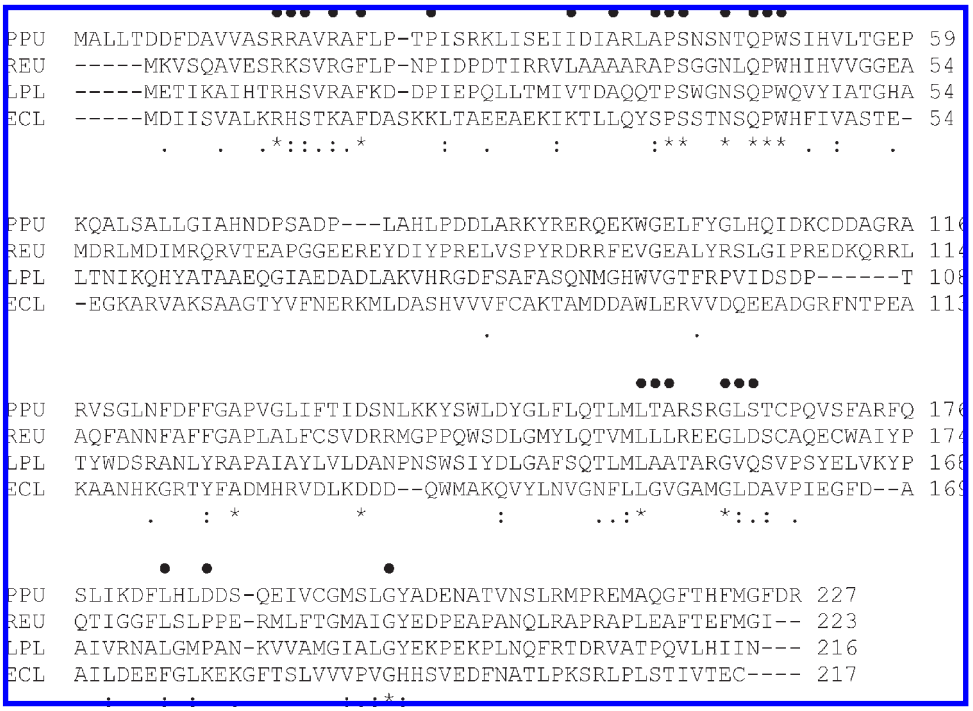

Figure 1. Comparison of PnbA reductase from *L. plantarum* WCFS1 (LPL) (accession no. CAD62742) and known PnbA reductases from *P. putida* (PPU) (AAG01540), *R. eutropha* (REU) (YP_297809), and *E. cloacae* (ECL) (Q01234). The ClustalW2 program was used to compare sequences. Conserved residues of the nitroreductase family (Pfam 00881) are indicated (•). Residues that are identical (∗), conserved (:), or semiconserved (.) in all sequences of the alignement are also indicated. Dashes represent gaps introduced to maximize similarities.

in a final volume of 0.5 mL. The activity of reductase was started by the addition of NADPH, and the reduction rate of cytochrome C at 550 nm was measured ($\varepsilon = 21$ mM^{-1} cm^{-1}). (c) Reduction of ferricyanide was measured by using a mixture containing 90 mM phosphate buffer, pH 7.4, 500 μM NADPH, 1 mM potassium ferricyanide ($K_3[Fe(CN)_6]$, and reductase enzyme (1−5 μg/mL protein). Reduction of ferricyanide was measured at 420 nm ($\varepsilon = 1020$ M^{-1} cm^{-1}). This assay was also used for determination of optimal pH using buffers adjusted at pH values between 2 and 9. (d) Reduction of NBT was monitored in a reaction mixture containing 80 mM phosphate buffer, pH 7.4, 500 μM NADPH, 200 μM NBT, and enzyme (1−5 μg/mL protein) in a final volume of 0.5 mL. The reaction was started with the addition of substrate and reduction monitored with time at 580 nm ($\varepsilon = 7$ mM^{-1} cm^{-1}).

Reduction of Nitroaromatics, Quinones, and Pyridinium Cations by *L. plantarum* PnbA Reductase. The activity of recombinant PnbA reductase from *L. plantarum* was assayed with different substrates, including a number of nitrocompounds, pyridinium cations, and quinones by two different methods: HPLC-DAD-MS and by following the spectrophotometric NADPH depletion. Thus, reaction mixtures with a final volume of 0.5 mL containing reductase enzyme (usually from 0.25 to 5 μg/mL protein), NADPH (500 μM), and the corresponding substrate (from 0 to 1 mM) in 100 mM buffer phosphate, pH 7.4, were mixed and incubated at room temperature for 3 min; the reaction was stopped with the addition of 10% 2 N HCl and subsequently injected into HPLC and HPLC-MS to quantify and identify metabolites of *L. plantarum* reductase and to calculate the reaction rates. 4-Nitrobenzoic acid, 2,4-dinitrobenzoic acid, 2-nitrobenzoic acid, 4-hydroxylaminobenzoic acid, 2,4-dinitrophenol, and 3-nitrobenzoic acid were tested as substrates for PnbA reductase. The corresponding controls without reductase and/or NADPH were also carried out. Kinetic values of K_m and V_{max} were determined by nonlinear regression analysis fitting to Michaelis−Menten curves of formation rates of *p*-hydroxylamine products as a function of the concentration of nitroaromatic substrates from 0 to 1 mM. Enzyme reaction rate was calculated as micromoles of hydroxylamine product per minute per milligram of protein. On the other hand, the activity of *L. plantarum* PnbA reductase was also measured by monitoring the decrease in absorbance of NADPH at 340 nm in a spectrophotometer for 6 min in the presence of various nitrocompounds, pyridinium cations or quinone as substrates. For that, reaction mixtures (0.5 mL) containing reductase enzyme (from 1 to 5 μg/mL), NADPH (300 μM), and substrate (50 or 100 μM) in 100 mM phosphate buffer, pH 7.4, were added to quartz cuvettes. The reaction was started by the addition of substrates, and the reduction rate was calculated as the micromoles of NADPH oxidized per minute per milligram of protein.

Reversed Phase HPLC Analysis. The analysis of enzymatic reaction media was performed by RP-HPLC with UV diode array and fluorescence detection using a HPLC 1050 (Hewlett-Packard) with a 1100 diode array detector (DAD) and a 1046A-fluorescence detector. A 150 mm × 3.9 mm i.d., 4 μm, Nova-Pak C18 column (Waters, Milford, MA) was used for chromatographic separation. Chromatographic conditions were as follows: 50 mM ammonium phosphate buffer, pH 3 (buffer A), and 20% of A in acetonitrile (buffer B). The gradient was programmed from 0% (100% A) to 32% B in 12 min and to 90% B at 15 min. The flow rate was 1 mL/min, the column temperature was 40 °C, and the injection volume was 20 μL. Absorbance detection was set at 280 nm (analysis of 4-hydroxylaminobenzoic acid) and 243 nm (analysis of 2-nitro-4-hydroxylaminobenzoic acid). Identification of compounds was performed by UV spectra, mass spectrometry, and co-injection with synthetic standards. For metabolite quantification, a calibration curve of synthesized 4-hydroxylaminobenzoic acid was constructed (0−50 μM), whereas for quantification of 2-nitro-4-hydroxylaminobenzoic acid, the response factor at 243 nm of a calibration curve of 2,4-dinitrobenzoic acid was used because both substrate and product exhibited similar spectra.

Identification by HPLC-ESI-MS. To carry out mass spectrometric identification, reaction media of *L. plantarum* PnbA reductase, nitroaromatics, and NADPH obtained as above were analyzed on a 150 × 2.1 mm i.d. Zorbax SB-C18, 5 μm, column (Agilent Technologies) by using a series 1100 HPLC-MSD (Hewlett-Packard) (electrospray positive and negative ion mode). Eluent A was acetic acid (0.5%), and eluent B was 20% of A in acetonitrile. The gradient was programmed from 0% B to 60% B at 20 min and 100% B at 25 min; the flow rate was 0.25 mL/min. $T = 40$ °C; mass range was 50−500 amu, flow gas = 11 L/min, $T^a = 330$ °C, and cone voltage was 55 V. The pressure of the nebulizer was 40 psi, and capillary voltage was 4000 V.

Spectrophotometric Studies. UV−vis spectra were recorded with a Beckman spectrophotometer. Samples with the purified reductase from

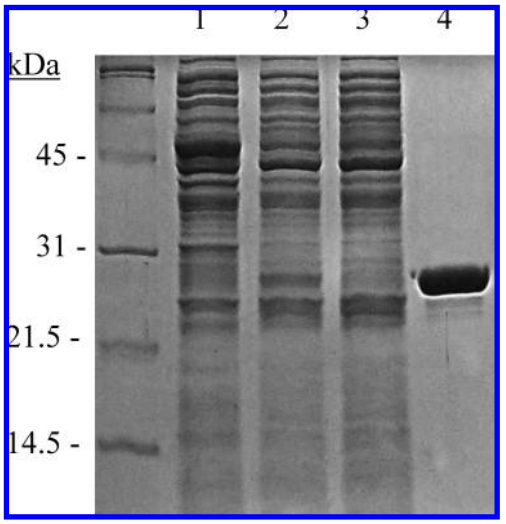

Figure 2. SDS-PAGE analysis showing the PnbA expression and PnbA purification from *L. plantarum* WCFS1 strain. Lanes: 1, soluble extract from *E. coli* JM109 (pURI3) cells; 2, extract from *E. coli* JM109 (pURI3-PnbA); 3, extract from *E. coli* JM109 (pURI3-PnbA) not retained on the affinity His-Trap-FF column; 4, purified PnbA reductase protein retained on the affinity column and eluted on 125 mM imidazole buffer. SDS−polyacrylamide gels were stained with Coomassie blue. The positions of some molecular mass markers (Bio-Rad) are indicated on the left.

L. plantarum were placed in quartz cuvettes, and spectra were determined in anaerobic conditions, before and after the addition of an excess of sodium dithionite ($Na_2S_2O_4$). The same apparatus was used for the determination of enzyme activities based on the decrease of NADPH (340 nm) in the presence of substrate at room temperature as indicated above.

RESULTS

Cloning, Expression, and Purification of *L. plantarum* PnbA. The deduced product of *L. plantarum pnbA* is a protein of 216 amino acid residues, 23.9 kDa, and p*I* of 6.65. This protein is included in the nitroreductase Pfam family (PF00881) that contains proteins involved in the reduction of nitrogen compounds (http://pfam.sanger.ac.uk/family?acc=PF00881). BLAST database searches of the translated *L. plantarum* DNA sequence showed high-scoring similarities (32−43% identity) with putative *p*-nitrobenzoate reductases (PnbA) from the sequenced lactic acid bacteria of *Lactobacillus* (*L. sakei*, *L. gasseri*, *L. reuteri*, *L. rhamnosus*, *L. jhonsonii*, and *L. delbrueckii*), *Lactococcus* (*L. lactis*), and *Pediococcus* (*P. pentosaceus*) genera, suggesting that these enzymes may exhibit similar functional properties in different lactic acid bacteria. The predicted sequence of the *L. plantarum* PnbA was aligned with selected nitrobenzoate reductases, the biochemical activity of which has been previously reported (**Figure 1**). From them, *L. plantarum* PnbA showed the highest overall identity (23%) with that of *Pseudomonas putida* and *Ralstonia eutropha*. *Enterobacter cloacae* PnbA showed only an 11% identity to *L. plantarum* protein. Other nitroreductases from enterobacteria such as *E. coli* and *S. typhimurium* also gave identities lower than 10%. In contrast, identities among nitroreductases from enterobacteria are higher than 80% (*44*). As deduced from **Figure 1**, all of these proteins showed some of the residues conserved in this family, belonging to the characteristic nitroreductase motif. A 2-nitroreductase enzyme from *Pseudomonas fluorescens* (*31*) not included in the alignment was only 3−7% identical to the other nitroreductases, and it did not present the conserved residues described for this family. In conclusion, on the basis of its amino acid sequence, it could be assumed that PnbA (lp_0050) from *L. plantarum* WCFS1 is a member of the nitroreductase family.

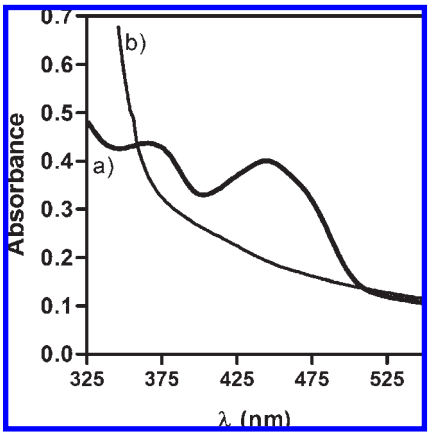

Figure 3. Absorption spectra of the purified flavoprotein PnbA from *L. plantarum* WCFS1 (a) and loss of the FMN bands following reduction with an excess of sodium dithionite (b).

To confirm that the *pnbA* (lp_0050) gene from *L. plantarum* WCFS1 encodes a functional *p*-nitrobenzoate reductase (PnbA), we expressed this gene in *E. coli* under the control of the T7 RNA polymerase-inducible Φ10 promoter. Cell extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3 vector plasmid alone did not show expression over the time course analyzed, whereas expression of an additional protein was apparent with cells harboring pURI3-pnbA (**Figure 2**). The molecular mass of the recombinant protein corresponded to that inferred from the nucleotide sequence (23.9 KDa). As the gene was cloned containing a purification poly-His tag, PnbA was purified on a His-Trap-FF crude chelating column and eluted with a stepwise gradient of imidazole. Highly purified PnbA protein was obtained from pURI3-pnbA (**Figure 2**), and fractions showing activity and usually eluting at 125 mM imidazole had yellow color. The presence of the protein band in the gel of the elution fractions, correlated well with the occurrence of reductase activity using DCPIP (dichlorophenolindophenol) as electron acceptor in presence of NAD(P)H. The presence of the His tag had no apparent effect on the catalytic activity. Thus, we could conclude that the *pnbA* (lp_0050) gene encoded a functional reductase in *L. plantarum* WCFS1. The eluted *L. plantarum* nitroreductase was dialyzed to eliminate the imidazole, and the purified protein was used for its biochemical characterization.

The purified PnbA reductase from *L. plantarum* was yellow, indicating the presence of a bound cofactor, and displayed UV−visible absorption spectra characteristic of flavin-containing enzymes (**Figure 3**). Thus, spectra of the protein showed two bands (maxima at 366 and 446 nm) resembling an oxidized FMN cofactor, which is usually present in bacterial nitroreductases. This profile quickly disappeared after reduction of the flavoenzyme with an excess of sodium dithionite as seen by the loss of absorbance at 446 nm and as expected for FMN enzymes. The enzyme appeared to lose part of the activity and color with time, suggesting that the coenzyme might not be tightly bound to the reductase (*25*). A further incubation of the protein with FMN appeared to partly recover activity (not shown).

Catalytic Properties of PnbA Reductase from *L. plantarum*. The catalytic properties of PnbA reductase from *L. plantarum* were studied with a number of electron acceptors in the presence of NAD(P)H as electron donor (**Table 1**). Nitroreductase from

Table 1. Reduction Rates of Several Electron Acceptors by PnbA Reductase from *Lactobacillus plantarum* WCFS1

electron acceptor	v^a (μmol/min/mg of protein)
DCPIP	6.6 ± 0.06
cytochrome C	0.2 ± 0.03
cytochrome C/menadione	1.51 ± 0.05
ferricyanide	158 ± 1.4
NBT	1.9 ± 0.09

[a] $v \pm$ SD. Assays were carried out as indicated under Materials and Methods and performed at least in triplicate.

L. plantarum reduced DCPIP (absorbance at 600 nm) in the presence of NADPH. It reduced cytochrome C in the presence of menadione, whereas in the absence of menadione as an electron carrier and acceptor it was a poor reductant (7.5 times lower) of cytochrome C, suggesting that this enzyme was not a cytochrome P450-like reductase. Moreover, *L. plantarum* PnbA reductase also reduced NBT to give formazan and was able to give electrons to the single electron acceptor ferricynanide. Therefore, various types of substrates were electron acceptors of *L. plantarum* PnbA reductase. As determined for the reduction of DCPIP, the enzyme could use both NADPH and NADH as electron donors, although using NADPH as reductant was 1.8 times more efficient than using NADH (500 μM). The optimal pH was measured in the range of 2−9, and the best reduction rate (reduction of ferricyanide) was achieved in the interval pH 5−7 (highest value around pH 6). The optimal temperature (reduction of DCPIP) was 25 °C, and the activity significantly decreased after incubation of the enzyme at temperatures higher than 37 °C for 20 min. Inhibition studies were carried out with dicoumarol and diphenyliodonium, which are inhibitors of quinone reductases and flavoproteins. Both compounds inhibited in a dose-dependent manner the PnbA reductase of *L. plantarum*. Dicoumarol inhibited the reduction of DCPIP with an IC_{50} of 0.15 mM, whereas diphenyliodonium (DPI) inhibited it with an IC_{50} of 5.5 mM. Inhibition by DPI was expected as it is an inhibitor of flavoproteins (*45*), whereas inhibition by dicoumarol appears to need higher concentrations than those reported for NADPH:quinone reductases (*46*).

As shown in **Table 1**, several electron acceptors of reductases were good substrates of *L. plantarum* PnbA reductase in the presence of NAD(P)H. Subsequently, a number of nitroaromatics, quinones, and naturally occurring and toxic pyridinium cations were studied as substrates and electron acceptors of this nitroreductase. The reduction rate was performed by monitoring the decrease of NADPH in the presence of the corresponding substrate (**Table 2**). The pyridinium cations, paraquat and 2-methyl-β-carbolinium cations, were not reduced significantly by PnbA reductase. However, the quinones, menadione and benzoquinone, were good substrates of this enzyme with benzoquinone showing 5 times more activity than menadione. Among a range of nitroaromatic compounds containing nitro groups in different positions in the benzenic ring, including nitrophenols, nitrobenzene, and nitrobenzoic acids, only two nitrocompounds, 4-nitrobenzoate and 2,4-dinitrobenzoate, were substrates of this enzyme as shown by an increased removal of NADPH (340 nm) when compared with a control in the absence of the nitroaromatic substrate. The activity for reduction of 2,4-dinitrobenzoic acid was 12-fold higher than for 4-nitrobenzoate, suggesting that the former was a better substrate for *L. plantarum* PnbA reductase. From these results only specific nitrocompounds could be reduced in the presence of NADPH by PnbA reductase.

Identification of Metabolites in the Reaction Mixtures by HPLC-MS. To characterize the reduction products of nitrocompounds, enzymatic reaction media containing *p*-nitrobenzoic acid, NADPH, and *L. plantarum* PnbA reductase were studied by HPLC-DAD and MS (electrospray) (**Figure 4**). *p*-Hydroxylaminobenzoic acid was identified as a major metabolite in the reaction media containing *p*-nitrobenzoic acid as shown by HPLC-MS, with ions at m/z 154 $(M + H)^+$, 137 $(M + 1 - 17)$ (positive ESI), and m/z 152 $(M - H)^-$ (negative ESI) (**Figure 4a**). This metabolite coeluted with a synthetic standard of *p*-hydroxylaminobenzoic acid and afforded similar absorption spectra (maximum at 278 nm). In the reaction mixtures, a trace peak of nitrosobenzoic acid (negative ESI) at m/z 150 $(M - H)^-$ also appeared, suggesting that it could be an intermediate in the reaction. In addition, traces of *p*-aminobenzoic acid eluting after the corresponding *p*-hydroxylamine compound (m/z at 136 $(M - H)^-$ and 138 $(M + H)^+$ in negative and positive ion modes) were detected. This amine product was not increased in the reaction media, suggesting that it was not a final product of PnbA reductase, and it might have appeared from chemical reduction. Indeed, no reduction was observed when synthetic *p*-hydroxylaminobenzoic acid was used as a substrate of PnbA reductase in the presence of NADPH. On the other hand, 2,4-dinitrobenzoic acid was also reduced in the reaction media by *L. plantarum* PnbA reductase, and a major metabolite was identified by HPLC-MS (**Figure 4b**) as *p*-hydroxylamino-2-nitrobenzoic acid in positive ESI at m/z 199 $(M + H)^+$ and 181 and in negative ESI at m/z 197 $(M - H)^-$ and 395 $(2M - H)^-$. The two substrates in **Figure 4** provided negative ESI at m/z 166 $(M - H)^-$ and 122 $(M - 45)$ (4-nitrobenzoic acid) and at m/z 211 $(M - H)^-$, 167 $(M - 45)^-$, and 423 $(2M - 1)^-$ (2,4-dinitrobenzoic acid).

Table 2. Activity of PnbA Reductase from *L. plantarum* WCFS1 Strain with Different Nitrocompounds, Pyridinium Cations, and Quinones as Substrates

substrate	v^a (μmol of NADPH/min/mg of protein)
1,4-benzoquinone	12.3 ± 3.0
menadione	2.26 ± 0.24
4-nitrobenzoic acid	2.9 ± 0.8
2,4-dinitrobenzoic acid	36.1 ± 6.1
2-nitrobenzoic acid	ND
3-nitrobenzoic acid	ND
2-hydroxy-3,5-dinitrobenzoic acid	ND
2,4-dinitrophenol	ND
2,4,6-trinitrophenol (picric acid)	ND
2-amino-4-nitrophenol	ND
nitrobenzene	ND
5-nitroindazole	ND
4-nitrophenyl-β-D-glucopyranoside	ND
N-(2-nitrophenyl)proline	ND
N-(2-nitrophenyl)pipecolic acid	ND
N-(2,4-dinitrophenyl)glycine	ND
N-(2-nitrophenyl)glycine	ND
1,1-dimethyl-1,4-bipyridyl (paraquat)	ND
2-methylnorharman cation	ND
2-methylharman cation	ND

[a] The enzymatic activity ($v \pm$ SD) was measured as indicated under Materials and Methods in the presence of enzyme (1−5 μg/mL), 300 μM NADPH, and 100 μM substrate with the exception of menadione and 2,4-dinitrophenol, which were assayed at 50 μM to avoid signal saturation. The rate was measured as the decrease of NAPDH (340 nm) in the first 1−2 min reaction at room temperature (25 °C), and the NADPH reduction in the absence of substrate was subtracted. ND, not detectable activity.

In agreement with previous spectrophotometric results (**Table 2**), no reduction was observed by HPLC(DAD)-MS of 2,4-dinitrophenol, 2-nitrobenzoic acid, or 3-nitrobenzoic acid, suggesting that PnbA reductase was selective for some specific nitrocompounds as electron acceptors and with regard to the position of the nitrosubstituent in the benzenic ring. The reduction of nitro in the para position with respect to the carboxylic group was required because

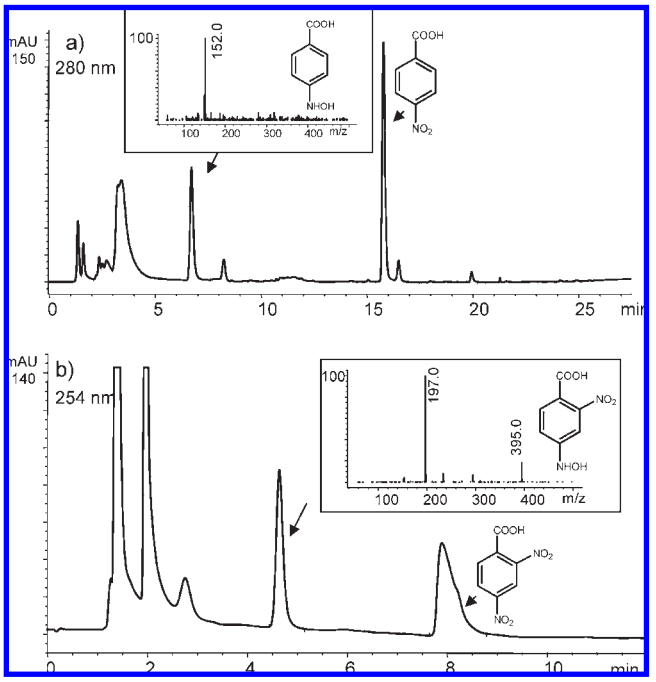

Figure 4. HPLC-MS (ESI) analysis of the reaction media of 4-nitrobenzoic acid (280 nm) (**a**) and 2,4-dinitrobenzoic acid (254 nm) (**b**) reduction with *L. plantarum* PnbA reductase. Electrospray (ESI) spectra in the negative ion mode of the corresponding hydroxylamine products are given. Reaction media in 100 mM phosphate buffer, pH 7.4, contained substrate (100 μM), NADPH (500 μM), and PnbA reductase (5 μg/mL) and were incubated for 10 min at room temperature (25 °C); the reaction was stopped as indicated under Material and Methods.

2- or 3-nitrobenzoic acid was not reduced by *L. plantarum* PnbA reductase. As mentioned above, 4-hydroxylaminobenzoic acid (100 μM) was not reduced to the corresponding amine by this reductase in the presence of NADPH, showing that the hydroxylamine was a final product of the catalytic reaction.

Enzyme Reduction Kinetics of PnbA Reductase from *L. plantarum*. The reduction of 4-nitrobenzoic acid in the presence of NADPH to give the corresponding hydroxylamine was rapid and linear in the first 10 min of incubation. Then, 3 min was subsequently used for kinetic studies by HPLC-DAD to calculate reaction rates and kinetic parameters. **Figure 5** shows the Michaelis−Menten curves with the experimental reaction rates as a function of the concentration of substrate both for 4-nitrobenzoic acid and for 2,4-dinitrobenzoic acid that gave 4-hydroxylaminobenzoic acid and 4-hydroxylamino-2-nitrobenzoic acid, respectively. **Table 3** gives the kinetic values of V_{max}, K_m, and the ratio V_{max}/K_m. The calculated V_{max} was 2.3 times higher for the reduction of 2,4-dinitrobenzoic acid than for 4-nitrobenzoic acid, whereas the K_m was much lower for 2,4-dinitrobenzoic acid (32-fold) than for 4-nitrobenzoate. This suggests a much higher affinity of *L. plantarum* reductase for 2,4-dinitrobenzoate than for 4-nitrobenzoate. Thus, V_{max}/K_m ratio was much higher for 2,4-dinitrobenzoate, indicating a higher suitability of this specific nitrocompound for the reduction by PnbA reductase. On the other hand, the reaction rate to give the *p*-hydroxylamine derivative from 4-nitrobenzoic acid was similar in aerobic and anaerobic conditions (not shown), suggesting that this reductase was not highly affected by the oxygen as expected for an oxygen-insensitive nitroreductase. By following the elimination of NADPH (absorbance) and the corresponding formation of hydroxylamine by HPLC, the stoichiometry calculated was about 2 mol of NADPH consumed per mole of hydroxylamine.

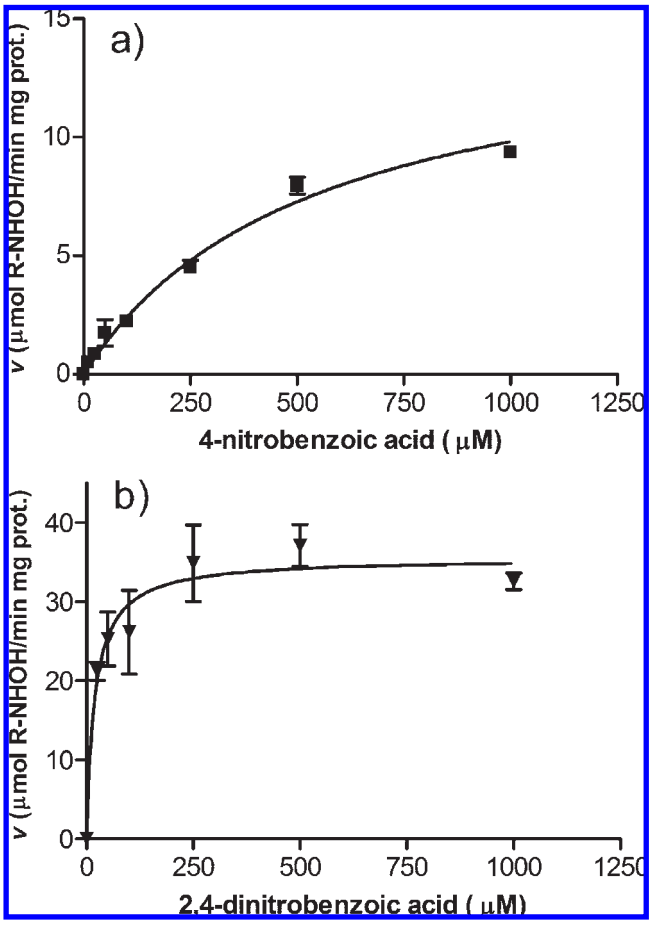

Figure 5. Michaelis−Menten curves of the enzymatic reduction of 4-nitrobenzoic acid (**a**) and 2,4-dinitrobenzoic acid (**b**) by PnbA reductase of *L. plantarum*. Reaction media contained 4-nitrobenzoic acid or 2,4-dinitrobenzoic acid in 100 mM phosphate buffer, pH 7.4, NADPH (500 μM), and PnbA reductase (0.25 μg/mL for 2,4-dinitrobenzoic acid and 2 μg/mL for 4-nitrobenzoic acid). Reaction was performed at room temperature for 3 min. Velocity (v) is given as micromoles of corresponding hydroxylamine product RNHOH per minute per milligram of PnbA reductase.

Table 3. Kinetic Parameters (V_{max} and K_m) of the Reduction of 4-Nitrobenzoic Acid and 2,4-Dinitrobenzoic Acid to the Corresponding Hydroxylamine Products by *L. plantarum* PnbA Reductase

substrate	K_m (μM) ± SE	V_{max} ± SE (μmol of product/min/mg of protein)	V_{max}/K_m (L min^{-1} mg of protein^{-1})
4-nitrobenzoic acid	536 ± 102	15.1 ± 1.6	0.03
2,4-dinitrobenzoic acid	16.7 ± 6.4	35.5 ± 2.0	2.13

DISCUSSION

In this research, the gene (lp_0050) encoding a functional nitrobenzoate reductase (Pnb A) from *L. plantarum* WCFS1 was cloned and expressed. This gene had been previously annotated as a PnbA reductase during its complete DNA sequencing (*3*). In addition, similar proteins annotated as PnbA reductases were found in the genomes of several lactic acid bacteria belonging to the *Lactobacillus* genera (*L. sakei*, *L. gasseri*, *L. reuteri*, *L. rhamnosus*, *L. jhonsonii*, and *L. delbrueckii*) as well as *Lactococcus* (*L. lactis*) and *Pediococcus* (*P. pentosaceus*) genera (*16*, *47*), showing a 32−43% identity to that of

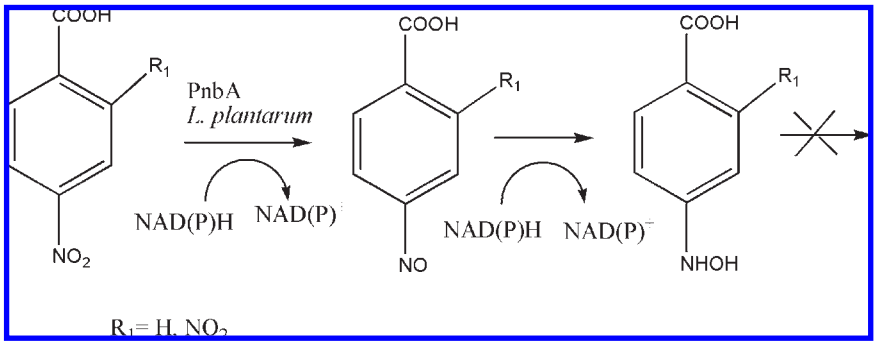

Figure 6. Selective reduction of 4-nitrobenzoic acid compounds to nitroso and hydroxylamine derivatives by PnbA reductase from *L. plantarum* WCFS1.

L. plantarum. However, none of these putative reductases had been previously cloned and/or biochemically characterized. The amino acid sequence of the PnbA reductase from *L. plantarum* showed a 23% identity to known nitroreductases from *P. putida* and *R. eutropha*, but less than 11% to nitroreductases from enterobacteria such as *E. cloacae*, *E. coli*, and *S. typhimurium*. A recombinant plasmid containing the *pnbA gen* was cloned into *E. coli*, and the encoded protein was shown to be a catalytically active *L. plantarum* PnbA reductase, resembling kinetic properties and inhibitor susceptibility of flavin reductases. This enzyme was a FMN-flavoprotein (yellow color) that used both NADPH and NADH as electron donors, and it was inhibited by dicoumarol and DPI. It could use a broad spectrum of electron acceptors such as DCPIP, NBT, ferricyanide, and quinones, suggesting that its active site could accommodate molecules of various sizes and structures as substrates. In contrast, it was more selective regarding the reduction of nitrocompounds, with only 4-nitrobenzoates being reduced, which suggests a higher selectivity for two electron reduction of nitroaromatic groups. Moreover, *L. plantarum* PnbA reductase was not able to reduce potentially toxic pyridinium cations such as the herbicide paraquat and the naturally occurring 2-methyl-β-carbolinium alkaloids. These compounds might generate free radicals and superoxide anion if reduced, contributing to their toxicity (*37*, *38*, *48*, *49*).

As occurs with other characterized nitroreductases, the natural substrates of *L. plantarum* nitroreductase and its physiological and/or metabolic role in lactic bacteria are unknown (*7*). Because NAD(P)H is used as an electron donor, it has been speculated that nitroreductases could link pyrimidine nucleotide oxidation to the reduction of substrates to maintain the steady supply of oxidized pyrimidine nucleotides. *L. plantarum* PnbA reductase has the ability to utilize a variety of electron acceptors such as quinones and other substrates; thus, it could remove oxidants, which otherwise would be reduced to a free radical state and form intermediate species reacting with DNA and proteins. This raises the possibility that nitroreductases could somehow protect cells against oxidative stress and the toxic effects caused by exposure to quinones and/or related cellular oxidants (*23*, *28*, *50*). On the other hand, nitroreductases could function as specific enzymes involved in degradation pathways of nitrocompounds and are of potential use in bioremediation of pollutants (*7*, *13*). These nitroreductases might be also of interest in the therapeutic action of antimicrobial and anticancer prodrugs (*10*, *12*, *51*).

Living organisms can utilize nitroaromatic compounds, which are metabolized by nitroreduction catalyzed by two types of nitroreductases: oxygen-sensitive and oxygen-insensitive (*9*, *15*). Oxygen-sensitive nitroreductases such as cytochrome P-450 oxidoreductase may catalyze the one-electron reduction of nitro to the anion free radical, which is reoxidized to the parent compound by O_2 in a futile redox cycle producing superoxide anion. Oxygen-insensitive nitroreductases such as NAD(P)H-quinone oxidoreductase and nitroreductases of enteric bacteria (*E. coli*, *E. cloacae*, *Salmonella*) catalyze the two-electron reduction of the nitro group to nitroso, hydroxylamine, and/or amine derivatives (*52*, *53*). The functional PnbA reductase from *L. plantarum* belongs to this latter family of nitroreductases and reduced *p*-nitrobenzoates (4-nitrobenzoate and 2,4-dinitrobenzoate) initially to 4-nitrosocompounds, which were rapidly converted to hydroxylamine derivatives as final products (**Figure 6**). The enzyme was not able to reduce the hydroxylamine further to *p*-aminobenzoate. This behavior agrees well with other microbial nitroreductases (*53*) that afford nitrosoaromatic as a limiting step that quickly reacts to give hydroxylamine products (*18*, *54*).

Nitroaromatic compounds constitute a wide range of chemicals having potent biological activity with significant human health and environmental implications. Many nitrocompounds are well-known as potent mutagens and carcinogens (*5*, *55*, *56*). The biological activity of these compounds is linked to their reductive metabolism catalyzed by nitroreductases to hydroxylamine, nitroso, and electrophilic species (*33*, *34*, *57*). Bacterial nitroreductases such as that of *L. plantarum* perform two-electron reduction of nitroaromatic compounds to hydroxylamine derivatives (*21*, *22*, *58*, *59*). They are distributed among different microorganisms, suggesting a certain relevance within microbial metabolic activities (*7*, *16*). Nevertheless, certain aspects of their catalytic process such as substrate specificity and reduction mechanism are not well understood. Thus, nitroreductases from *E. cloacae* (*21*), *S. typhimurium* (*25*), *E. coli* (*23*, *24*, *60*), *Ralstonia eutropha* (*18*), *Rhodobacter capsulatus* (*19*, *61*), and *Pseudomonas* (*22*, *32*, *62*) are able to reduce a wide range of nitroaromatics including nitroalkylbenzenes, nitrophenols, nitrobenzoates, nitrofurazone, and *p*-nitroacetophenone, among others. Then, they appear to be relaxed on substrate specificity and can reduce nitrocompounds and other electron acceptors such as quinones (*23*, *58*, *63*). In this regard, the PnbA reductase from *L. plantarum* was selective for the reduction of nitrocompounds and reduced only those with a 4-nitrobenzoate moiety such as 4-nitrobenzoate and 2,4-dinitrobenzoate. The efficiency (V_{max}/K_m) for the reduction of 2,4-dinitrobenzoate was much higher than that for 4-nitrobenzoate, whereas it was unable to reduce other nitrobenzoates or nitrophenols. This might suggest that this PnbA reductase, and perhaps other nitroreductases annotated in lactic acid bacteria (*16*, *47*), may form a class of more selective 4-nitrobenzoate nitroreductases. Moreover, this reductase might have similar functional properties in different lactic acid bacteria as it shows a high-scoring similarity (65−72% similarity) to other putative PnbA reductases from lactic acid bacteria.

In conclusion, we have cloned, expressed, and characterized a novel *p*-nitrobenzoate (PnbA) reductase from *L. plantarum* WCFS1 that was previously annotated on its complete genome

sequence. Similar proteins seem to be present in several other lactic acid bacteria. The PnbA reductase of *L. plantarum* utilized a wide range of electron acceptors including quinones, DCPIP, NBT, and ferricyanide, but it was not able to reduce pyridinium cations such as paraquat and 2-methyl-β-carbolinium substances. However, it was selective for nitrocompounds, reducing only 4-nitrobenzoates (4-nitrobenzoate and 2,4-dinitrobenzoate) among a number of nitrocompounds assayed. This PnbA nitroreductase may be distinct from other previously described bacterial nitroreductases because of its selectivity for 4-nitrobenzoates. This different selectivity could be due to the fact that *L. plantarum* PnbA reductase exhibits a 23% identity to nitroreductases from *P. putida* and *R. eutropha* and was even less identical (< 11%) to enterobacteria PnbA (e.g., *E. cloacae*, *E. coli*, and *S. typhimurium*. This is the first characterization of a nitroreductase in lactic acid bacteria that widely occur in foods, the environment, and the human intestinal tract, and thereby these results might be of further interest.

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