The Homogentisate and Homoprotocatechuate Central Pathways Are Involved in 3- and 4-Hydroxyphenylacetate Degradation by Burkholderia xenovorans LB400

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Abstract

**Background:** Genome characterization of the model PCB-degrading bacterium *Burkholderia xenovorans* LB400 revealed the presence of eleven central pathways for aromatic compounds degradation, among them, the homogentisate and the homoprotocatechuate pathways. However, the functionality of these central pathways in strain LB400 has not been assessed and related peripheral pathways has not been described.

**Methodology/Principal Findings:** The aims of this study were to determine the functionality of the homogentisate and homoprotocatechuate central pathways in *B. xenovorans* LB400 and to establish their role in 3-hydroxyphenylacetate (3-HPA) and 4-hydroxyphenylacetate (4-HPA) catabolism. Strain LB400 was able to grow using 3-HPA and 4-HPA as sole carbon source. A genomic search in LB400 suggested the presence of *mhaAB* and *hpaBC* genes clusters encoding proteins of the 3-hydroxyphenylacetate and 4-hydroxyphenylacetate peripheral pathways. LB400 cells grown with 3-HPA and 4-HPA degraded homogentisate and homoprotocatechuate and showed homogentisate 1,2-dioxygenase and homoprotocatechu-2,3-dioxygenase activities. Transcriptional analyses by RT-PCR showed the expression of two chromosomally-encoded homogentisate dioxygenases (BxeA2725 and BxeA3900) and the *hpaD* gene encoding the homoprotocatechuate 2,3-dioxygenase during 3-HPA and 4-HPA degradation. The proteome analyses by two-dimensional polyacrilamide gel electrophoresis of *B. xenovorans* LB400 grown in 3-HPA and 4-HPA showed the induction of fumarylacetoacetate hydrolase HmgB (BxeA3899).

**Conclusions/Significance:** This study revealed that strain LB400 used both homogentisate and homoprotocatechuate ring-cleavage pathways for 3- hydroxyphenylacetate and 4-hydroxyphenylacetate catabolism and that these four catabolic routes are functional, confirming the metabolic versatility of *B. xenovorans* LB400.

Introduction

Aromatic compounds are widely distributed in ecosystems mainly released from plant materials and by anthropogenic activities. Bacteria have evolved to metabolize diverse aromatic compounds including environmental pollutants, using these compounds as carbon and energy source. A broad range of aromatic compounds is degraded by bacteria through peripheral pathways that funnel into few central pathways [1–5]. In peripheral pathways, activation of the aromatic ring is commonly mediated by monoxygenases or dioxygenases that produce dihydroxylated intermediates. Central pathways involve the fission by a dioxygenase of the dihydroxylated aromatic metabolic intermediates at ortho- or meta-position and lead to the formation of Krebs cycle intermediates [1–4]. Bacterial metabolism of aromatic compounds has been extensively studied [3–11]. The metabolic reconstruction of aromatic compounds pathways in model environmental bacteria has been achieved by genome sequence analyses [3,12–14]. However, further studies are needed to establish gene function and to elucidate functional aromatic metabolic pathways of bacteria.

*B. xenovorans* LB400 is a model bacterium for the degradation of PCBs and other aromatic compounds [3,15,16]. Strain LB400 has one of the largest bacterial genomes (9.73 Mbp), distributed in a major chromosome (C1), a minor chromosome (C2) and a megaplasmid (MP). Genome analyses of strain LB400 revealed the presence of genes encoding eleven central pathways and twenty peripheral pathways for aromatic compounds degradation [3]. The genes encoding the homogentisate and the homoprotocatechuate central pathways were identified in *B. xenovorans* LB400 genome [3]. The *hmgABC* and the *hpaGEDFHI* gene clusters are located at C1 and C2, respectively. Additional *hmg* gene copies were identified within LB400 genome [3]. Both ring-cleavage pathways are used in aromatic amino acid metabolism in *Bacteria* and *Eucarya* [17–20].
The homogentisate pathway has been described as the central route for the degradation of L-tyrosine and L-phenylalanine in bacteria, fungi and mammals [19,21,22]. The homogenisate central pathway is encoded by the hmgABC operon in Pseudomonas putida strain U [19]. Homogenisate (HMG) cleavage is catalysed by the HMG 1,2-dioxygenase HMG-A producing maleylacetocetate (MA). MA is isomerized by a maleylacetate isomerase (HmgC) into fumarylacetocetate, which is further converted by a fumarylacetocetate hydrolase (HmgB) into fumarate and acetocetate [23]. The homoprotocatechuate pathway has been described as a central route for the catabolism of aromatic amino acids and related compounds in Klebsiella pneumoniae, P. putida, and Escherichia coli [2,24–28]. The homoprotocatechuate (HPC) central pathway is encoded by the hpaD gene. The product 5-carboxymethyl-2-hydroxymuconic semialdehyde (CHMS) is then converted to 5-carboxymethyl-2-hydroxy-muconic acid (CHM) and subsequently degraded by the hpaF, hpaG, hpaI and hpaD gene products into Krebs cycle intermediates [21,17,18,28].

Degradation of phenylacetate hydroxylated-derivatives is channelled into the HMG or the HPC central pathways [17,20,29,30]. Assimilation of 3-hydroxyphenylacetate (3-HPA) through the homogenisate pathway in P. putida U has been reported. The mhaAB genes encode a 3-HPA 6-hydroxylase that hydrogenates 3-HPA at C-6 producing HMG [19,30]. In addition, P. putida strain U degrades 4-hydroxyphenylacetate (4-HPA) via the homoprotocatechuate pathway [27]. E. coli strains W, B and C are able to degrade both 3-HPA and 4-HPA via the homoprotocatechuate central route, which is encoded by the inducible hpaGEDFI operon [2,18,24,26]. The hpaD gene encodes a 4-HPA 3-hydroxylase involved in the hydroxylation of 4-HPA and 3-HPA to yield HPC [18]. The hpaBC and hpaGEDFI gene clusters constitute a single operon in E. coli strain W [2,18].

The aims of this study were to determine functional homogenisate and homoprotocatechuate central pathways in B. xenovorans strain LB400 and to establish their role in 3-HPA and 4-HPA peripheral pathways, which has not been described previously in this bacterium. This study showed that both homogenisate and homoprotocatechuate central pathways are involved in 3-HPA and 4-HPA degradation in strain LB400.

Materials and Methods

Chemicals

3-hydroxyphenylacetic acid (>99% purity), 4-hydroxyphenylacetic acid (98% purity) and 3,4-dihydroxyphenylacetic acid (homoprotocatechuate; 98% purity) and 2,5-dihydroxyphenylacetic acid (homogenisate; 98% purity) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Bacterial strain and culture conditions

Burkholderia xenovorans LB400 was cultivated at 30°C in mineral M9 medium with trace solution and glucose (5 mM) [31], 3-HPA (5 mM) or 4-HPA (5 mM) as the sole carbon and energy source. Growth was determined by measuring turbidity at 525 nm and by counting colony-forming units (CFU). Aliquots taken from cultures were diluted and plated on Luria-Bertani agar medium. CFU per milliliter values were calculated as the mean ± SD of at least three independent experiments.

Resting cell assays

Resting cells (turbidity 525nm = 2.0) in 50 mM sodium phosphate buffer (pH 7.0) were incubated with HMG or HPC (0.03 mM) at 30°C. Aliquots of cell suspensions were taken at different incubation times and centrifuged (19,203 g for 2 min). Assays with boiled cells and without cells were used as controls. Cell-free supernatants were analyzed, using a Waters liquid chromatograph model 515 equipped with a UV detector and a RP-C18/Chromspher 5-μm column (Supelco, Bellefonte, USA). The mobile phase contained 20% acetonitrile, 20% methanol, 60% water and 0.1% phosphoric acid. The flow rate was 0.5 mL min⁻¹. HMG and HPC had retention times of 4.6 and 5.1 min, respectively. HMG and HPC were quantified using calibration curves with authentic standards. Resting cells experiments were performed in triplicate.

Preparation of cell extracts

Cells harvested at exponential phase of growth (turbidity525 nm = 0.40–0.45) were centrifuged (10,733 g for 10 min) at 4°C and washed with 50 mM sodium phosphate buffer (pH 7.0). Bacterial cells were disrupted using an ultrasonic cell disruptor MicrosonTM at 4°C. Lysate was clarified by centrifugation (19,300 g for 15 min) at 4°C. Protein concentration was determined using Qubit™ fluorometer (Invitrogen).

Dioxygenase activity assays

HMG dioxygenase activity was determined spectrophotometrically by measuring the formation of MA at 330 nm as previously described [32]. The assay volume (1 ml) contained: 50 mM phosphate buffer (pH 7.0), 50 μM FeSO₄, 2 mM ascorbate, crude extract (100 μg of protein) and 0.3 mM HMG. The reactions were carried out at 30°C and initiated by the addition of HMG. One enzyme milliunit corresponds to the transformation of 1 μmol of HMG to MA per min at 30°C under the conditions described above. HMG dioxygenase activity was calculated using the molar extinction coefficient of MA, 13,500 M⁻¹ cm⁻¹ [33]. HPC dioxygenase activity was measured by monitoring the formation of 5-carboxymethyl-2-hydroxymuconate semialdehyde (CHMS) at 580 nm [34]. LB400 cells cultivated using 3-HPA, 4-HPA or glucose were harvested at a turbidity525 nm of 0.45, washed and concentrated. HPC dioxygenase activity of LB400 cell suspension (turbidity525 nm = 2.0) was determined by incubation with 0.3 mM HPC at 30°C. At intervals, the A380 values of supernatants were measured.

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

2-DE gels with non-equilibrium pH gradient electrophoresis (pH 3–10) were performed as previously described [31,35]. Cell cultures were grown using glucose, 3-HPA or 4-HPA (5 mM) as sole carbon source. Cells were harvested at exponential phase (turbidity525 nm = 0.40–0.45) by centrifugation, disrupted with a sonicator and concentrated in a speed-vac concentrator. Dried samples were suspended in lysis buffer (9.5 M urea, 2% w/v IGE-PAL CA-630, 2% amphotolites (1.6% ampholitès pH 5–7, 0.4% ampholitès pH 3–10, Bio-Rad) and 5% β-mercaptoethanol. NEPHGE gels were electrophoresed for 6.5 h at 400 V. The second dimension was performed in 11% polyacrylamide-SDS gels. Proteins were visualized with Coomassie brilliant blue R-250. The volume (Int × mm²) of the spots was analyzed, using Quantity One image analysis software (Bio-Rad Laboratories). To standardize the quantification of independent experiments, the ratio of the intensity of the protein to a reference protein was calculated.

Protein sequencing and identification

To perform the mass spectrometric analysis, protein spots were recovered from gels and prepared as described [31]. For
matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis, the peptide extracts were analysed with a MALDI-TOF UltraNex apparatus (Bruker, Bremen, Germany). The peptide fingerprints obtained by mass spectrometry MALDI-TOF were used for searches in the NCBI protein database with the Matrix science MASCOT search tool. The complete sequences of each protein were obtained and BLAST searches were executed with the FASTA tool for the identification and similarity data analyses.

Isolation of total RNA and RT-PCR

Total RNA was isolated from LB400 cells in the stationary growth phase using an RNaseasy mini kit (Qiagen, Hilden, Germany) according to the manufacturers’ recommendations. DNase I treatment was carried out using the RNase-Free DNase Set (Qiagen, Hilden, Germany) to degrade any residual DNA. The RNA concentration was quantified using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA). Reverse transcription-PCR (RT-PCR) was carried out with sequence-specific primers design (Invitrogen, Carlsbad, CA, USA). The BxeA2725 gene was used as control for DNA contamination using the primers CTCGCGTTGGCAGCAAAAGTG-3 and CGAAATTCTCGCAGATGTA-3. Reverse transcriptase reaction of the RNA was carried out using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA). The BxeA2725 gene (hmgA1) was amplified using the primers HmgA1-f (5’-ATT-TTGCAACCCAGGCTGGC-3’) and HmgA1-r (5’-CGACGGTGTAAAAGTGGTCC-3’). Amplification of the BxeA3900 gene (hmgA2) was carried out with specific primers HmgA2-f (5’-ATCTGCGAACAACATGTCAGTTCC-3’) and HmgA2-r (5’-GCCCGGAATTCTCTCCGAGATGTA-3’). Reverse transcriptase reaction for hpaD mRNA was performed with the primers HpaD-f (5’-CTCGCGTTGGCAGCAAAAGTG-3’) and HpaD-r (5’-GCGGAGGCCGTAACCCGGAAG-3’). Amplification of the 16S rRNA gene was used as control for DNA contamination using the primers 27f (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492r (5’-TACGGYTAC CTTGTTACGACTT-3’).

Results

Analysis of hmg and hpa gene clusters in strain LB400

The hmgABC gene cluster (BxeA2725; BxeA2724 and BxeA2723; hereafter hmgA, hmgB1 and hmgC1, respectively) is located at C1 of strain LB400 genome. Copies of the hmgA1 gene and hmgB1 genes (BxeA3900 and BxeA3899, respectively) were identified in a 1,300 kb distant region from hmgA1B1C1 cluster at C1 (hereafter hmgA2 and hmgB2). A hmgC gene copy (BxeA1411; hereafter hmgC2) is located in a different region of C1 [3] (Fig. 1A). Sequence analyses of the chromosomal region flanking the hmgA1B1C1 gene cluster in the strain LB400, revealed that the products of the open reading frames (ORFs) BxeA2727 and BxeA2726 shared 47% and 35% sequences identity with the MhaA and MhaB proteins from P. putida strain U, respectively [30] (Fig. 1A). In P. putida U the enzyme 3-HPA 6-hydroxylase (MhaAB) is a two-component FAD-dependent monooxygenase that hydroxylates 3-HPA to produce HMG (Fig. 2). The large component MhaA is a flavoprotein and the small component MhaB is a coupling protein [30]. In this study, the presence of the mhaAB genes located adjacent of hmgA1B1C1 gene cluster in LB400 was revealed, suggesting a role of mhaAB genes in 3-HPA peripheral reaction leading to HMG.

The gene cluster hpaG1G2EDFHII encoding the homoprotocatechuate pathway is located at C2 in strain LB400 genome (BxeB2028, BxeB2029, BxeB2030, BxeB2031, BxeB2032, BxeB2033 and BxeB2034) (Fig. 1B). Downstream and adjacent to the hpa gene cluster, a divergent gene putatively encoding the HpaR repressor protein of the HPC catabolic pathway was identified (Fig. 1B) [3]. Genes encoding the 4-HPA catabolic pathway were not found in the neighborhood of hpa gene cluster in strain LB400. The degradation of 4-HPA in E. coli strain W involves enzymes encoded in the 4-HPA hydroxylase hpaBC operon and the meta-cleavage operon hpaGEDFHII [18]. The hpaBC cluster of strain W encodes a two-component protein responsible for 4-HPA hydroxylation to yield HPC [18,29]. The BxeB2309 gene product of strain LB400 was identified as the 4-HPA 3-monoxygenase coupling protein HpaC located downstream to the hpa cluster (at 390 kb). The protein encoded by the BxeB2308 gene is highly related to the HpaB proteins from B. cenocepacia strain HI2424 and R. jostii RHA1 (Fig. 2). Based on its relatedness with FAD-dependent monooxygenases and its location next to a putative hpaC gene, the gene BxeB2308 may encode the main component of the closely related 4-HPA 3-monoxygenase (HpaB). However, further analyses are required to establish the 4-HPA 3-monoxygenase peripheral pathway encoded by BxeB2308 and BxeB2309 genes in LB400. The presence of genes

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**Figure 1.** Organization of genes encoding the homogentisate and homoprotocatechuate central pathways and related peripheral pathways in *B. xenovorans* LB400. A. The hmg genes encoding the homogentisate central pathway, and *mha* genes encoding a 3-HPA peripheral pathway located in the major chromosome (C1). B. The *hpa* cluster encoding the homoprotocatechuate central pathway, and *hpaBC* genes encoding a 4-HPA peripheral pathway located in the minor chromosome (C2) of strain LB400. Genes encoding ring-cleavage dioxygenases are indicated with black arrows. doi:10.1371/journal.pone.0017583.g001
putatively encoding peripheral reactions leading the homogentisate and homoprotocatechuate central pathways suggested functional HPA peripheral pathways leading to these ring-cleavage pathways in strain LB400.

Strain LB400 growth on HPAs

Sequence analyses of strain LB400 genome revealed the presence of putative genes encoding 3-HPA and 4-HPA peripheral pathways. To this end, the growth of strain LB400 on 3-HPA and
4-HPA as sole carbon source was studied. Strain LB400 was able to grow using 3-HPA and 4-HPA as sole carbon and energy source reaching a high biomass (data not shown). Strain LB400 attained at stationary phase (25 h), slightly higher biomass using 4-HPA (3.21 ± 10^7 CFU mL^-1) than using 3-HPA (1.28 ± 10^7 CFU mL^-1) and glucose (2.09 ± 10^7 CFU mL^-1) as sole carbon source. The growth on HPAs indicate that strain LB400 possess functional 3-HPA and 4-HPA catabolic pathways.

HMG and HPC degradation by strain LB400

In order to determine the functionality of the homogentisate and homoprotocatechuate central pathways, the degradation of these compounds was analysed. HMG and HPC degradation by strain LB400 were studied using resting cell assays. LB400 cells grown either in 3-HPA or 4-HPA were able to degrade HMG and HPC compounds. Cells grown in 3-HPA and 4-HPA degraded after 10 h of incubation 100% and >95% of HMG, respectively (Fig. 3A). It is worth noting that, during HMG degradation assays, a brown colored medium was only observed in control assays with boiled cells. In contrast, no pigmented medium was observed in assays with metabolically active cells. The brown pigmentation of the medium has been reported in bacteria and fungi that lack the homogentisate pathway, and thus HMG accumulation and its chemical oxidation were observed [19,20,37]. LB400 cells grown in 3-HPA and 4-HPA degraded after 22 h almost completely HPC (Fig. 3B). A slightly faster degradation of HPC was observed with 3-HPA-grown cells than with 4-HPA-grown cells. These results showed that strain LB400 degraded HMG and HPC, indicating that the corresponding ring-cleavage pathways are active in 3-HPA- and 4-HPA-grown cells and are involved in their degradation.

HMG and HPC ring-cleavage activities

To further determine functional homogentisate and homoprotocatechuate central pathways, the enzymatic activities of HMG dioxygenase and HPC dioxygenase were determined. HMG dioxygenase activity was measured in crude extracts of LB400 cells cultured in 3-HPA, 4-HPA and glucose. A high HMG dioxygenase activity was observed during growth on 3-HPA (82 mU/mg protein) and 4-HPA (75 mU/mg protein) (Fig. 4A). HMG dioxygenase activity was slightly higher on 3-HPA-grown cells than on 4-HPA-grown cells, whereas no activity was observed in glucose-grown cells. HPC dioxygenase activity was measured by monitoring the formation of CHMS after whole cells incubation with HPC. Cells grown on 3-HPA and 4-HPA showed high HPC dioxygenase activity. A moderately higher HPC dioxygenase activity was observed in 4-HPA-grown cells than in 3-HPA-grown cells. In contrast, a low HPC dioxygenase activity was measured in glucose grown cells (Fig. 4B). These results indicate an induction of the key enzymes of the homogentisate and homoprotocatechuate central pathways during growth of strain LB400 on 3-HPA and 4-HPA.

hmgA and hpaD expression analyses by RT-PCR

Further analyses were conducted in order to determine the role of the homogentisate and homoprotocatechuate pathways in 3-HPA and 4-HPA catabolism. The expression of hmgA and hpaD genes encoding the key enzymes HMG and HPC dioxygenases was analyzed during exponential growth of strain LB400 in 3-HPA, 4-HPA and glucose. Additionally, transcriptional analyses of two gene copies encoding HMG 1,2-dioxygenase were performed using specific primers for hmgA1 and hmgA2 genes. Expression of the hmgA1 gene was observed at early exponential growth phase of strain LB400 on 3-HPA, whereas no expression was detected during growth on 4-HPA or glucose (Fig. 5A). This indicates that hmgA1 gene is up regulated by 3-HPA in strain LB400. On the other hand, expression of the hmgA2 gene was detected in 3-HPA, 4-HPA and glucose grown-cells. These data also suggests lower hmgA2 transcription in glucose-grown cells (Fig. 5A).

Expression analysis of hpaD gene encoding HPC 2,3-dioxygenase was performed. The hpaD transcripts were detected at early
exponential phase of strain LB400 during growth on 3-HPA, 4-HPA and glucose as sole carbon source (Fig. 5B). Higher hpaD expression during growth on 3-HPA and 4-HPA was observed. These results indicate an expression of the dioxygenase-encoding genes during 3-HPA and 4-HPA catabolism in strain LB400. Furthermore, these results indicate that during 3-HPA degradation, two HMG 1,2-dioxygenase encoding hmgA genes are expressed.

Proteomic analyses during 3-HPA and 4-HPA degradation

A proteomic analysis of LB400 cells grown in 3-HPA and 4-HPA as sole carbon source was performed in order to elucidate catabolic enzymes involved in HPAs degradation. 2-DE analysis revealed the induction of an enzyme from the homogentisate central pathway measured by maleylacetate (MA) formation in crude extracts; B, HPC dioxygenase activity measured 5-carboxymethyl-2-hydroxy-muconic semialdehyde (CHMS) product formation in resting cells of strain LB400. doi:10.1371/journal.pone.0017583.g004

These results indicate an expression of the dioxygenase-encoding genes during 3-HPA and 4-HPA catabolism in strain LB400. Furthermore, these results indicate that during 3-HPA degradation, two HMG 1,2-dioxygenase encoding hmgA genes are expressed.

Proteomic analyses during 3-HPA and 4-HPA degradation

A proteomic analysis of LB400 cells grown in 3-HPA and 4-HPA as sole carbon source was performed in order to elucidate catabolic enzymes involved in HPAs degradation. 2-DE analysis revealed the induction of an enzyme from the homogentisate central pathway during growth of strain LB400 in 3-HPA and 4-HPA (Fig. 6). The polypeptide was identified by MALDI-TOF as fumarylacetate hydrolase HmgB, which catalyzes the conversion of fumarylacetate into fumarate and acetocetate. The protein sequence possesses 91% identity with HmgB protein from Burkholderia phytofirmans P3JN. The induction of HmgB during growth on 3-HPA, was 3.5-fold compared to glucose-grown cells. During growth on 4-HPA, induction of this protein was 2.5-fold compared to cells grown on glucose. The corresponding hmgB2 gene (BxeA3899) is adjacent to hmgI2 in strain LB400 genome. This result correlates with the expression of hmgA2 gene observed under the same growth conditions by RT-PCR. The induction of an enzyme belonging to the homogentisate ring-cleavage pathway is in accordance with the degradation of HMG and the increased HMG dioxygenase activity in strain LB400 during growth in 3-HPA and 4-HPA. Inducible expression of two enzymes from the homogentisate pathway observed during growth of strain LB400 in HPAs indicate that this catabolic pathway is involved in 3-HPA and 4-HPA degradation by strain LB400. The lower synthesis of HmgB protein in B. xenovorans LB400 grown in glucose indicates a basal expression of the catabolic hmgB1 gene during growth on this carbon source.

Figure 5. Expression of hmgA and hpaD genes during growth on HPAs. LB400 cells were grown on glucose (lanes 1) 3-HPA (lanes 2) and 4-HPA (lanes 3) as sole carbon source. A, Expression of hmgA1 (BxeA2725); B, expression of hmgA2 (BxeA3900); C, expression of hpaD. RT-PCR assays were performed using RNA from LB400 cells collected at early exponential growth phase. doi:10.1371/journal.pone.0017583.g005

Figure 6. Induction of fumarylacetate hydrolase HmgB in B. xenovorans LB400 during growth on HPAs. Cells were grown on glucose 5 mM (A), 3-HPA 5 mM (B) and 4HPA 5 mM (C). Proteins were separated by 2-D gel electrophoresis and stained with Coomassie blue. A segment of each 2-D gel is shown. doi:10.1371/journal.pone.0017583.g006
Discussion

This study has shown that the homogenitase and the homoprotocatechuate central pathways are involved in 3-HPA and 4-HPA catabolism by *B. xenovorans* strain LB400. 3-HPA and 4-HPA isomers are used by *B. xenovorans* LB400 as sole carbon and energy source for growth, indicating active peripheral and central catabolic pathways. Bacterial catabolism of hydroxyphenylacetates, particularly 3-HPA and 4-HPA compounds have been described to channel either the homogenitase or the homoprotocatechuate central pathways. Interestingly, this report showed that LB400 growth on 3-HPA and 4-HPA separately funnels into both central pathways. Similarly, in *P. putida* strain U, different catabolic pathways were reported for 4-HPA and 3-HPA degradation. It is worth noting that strain LB400 showed a faster growth in 3-HPA (5 mM) than *P. putida* strain U in 3-HPA (10 mM) [30]. In this study, LB400 reached high biomass using 4-HPA as sole carbon source (turbidity$_{525 \text{ nm}}$ of 1.1) (data not shown). In contrast, in similar media with 4-HPA as carbon source *P. putida* U attains lower biomass (turbidity$_{540 \text{ nm}}$ of 0.3) [27]. The MhaAB protein from strain U is highly specific for 3-HPA substrate to yield HMG [30], which is further degraded by the hmgABC gene products [19,30]. Interestingly, upstream to the hmgABC gene cluster in the LB400 genome, two ORFs (BxeA2727 and BxeA2726) with high identity to the mhaAB genes from strain U were found. The MhaA flavoprotein component (566 aa) and the MhaB coupling protein (60 aa) possess a similar length to the aminoacid sequence deduced from BxeA2727 (560 aa) and BxeA2726 (60 aa) genes of strain LB400. In a previous study, the BxeA2727 was annotated as a putative 3-3-hydroxy-phenylpropionate hydroxylase (MhpA) [3]. We propose that BxeA2727 and BxeA2726 of strain LB400 encode the two components of the hydroxylating enzyme MhaAB that is involved in catabolism of 3-HPA via HMG (Fig. 7A). However, further analyses are required to confirm the involvement of the mhaAB genes in 3-HPA degradation by strain LB400.

Alternatively, 4-HPA degradation in *P. putida* strain U is mediated by a 4-HPA 3-hydroxylase yielding HPC [27]. Interestingly, the results presented above indicate that both 4-HPA and 3-HPA compounds are degraded via HPC in strain LB400. However, based on hmgA and hpaD gene expression analyses and HMG and HPC dioxygenase activities, we postulate that 3-HPA in strain LB400 is preferentially channelled into the homogenitase central pathway. A previous report of 4-HPA catabolism showed that 4-HPA 3-hydroxylase from *E. coli* strain W hydroxylates 4-HPA and also 3-HPA yielding HPC [29]. A search of hpaD and hpaC genes in LB400 genome showed a gene (BxeB2309) with high identity to the hpaC gene encoding the 4-HPA 3-hydroxylase coupling protein of *E. coli* strain W located 309 b distant from the hpaGEDFH1 gene cluster in strain LB400. The adjacent gene (BxeB2308) probably encodes a FAD-dependent monooxygenase distant related to the HpaB hydroxylase component from *E. coli* W [29]. We propose that the BxeB2308 and BxeB2309 genes encode the two components of a hydroxylase involved in 3-HPA and 4-HPA catabolism (Fig. 7B).

Additionally, this study demonstrated that both chromosomally encoded homogenitase and homoprotocatechuate central pathways are functional in strain LB400. Resting cell assays indicated that strain LB400 degraded HMG and HPC compounds. Moreover, a high HMG dioxygenase activity was measured in 3-HPA and 4-HPA-grown cells (82 and 75 mU/mg of protein, respectively), whereas no activity was detected in glucose-grown cells (Fig. 4A). A similar HMG dioxygenase activity (68 mU/mg of protein) was previously reported in crude extracts of *P. putida* U cells grown on tyrosine or phenylalanine [19]. LB400 cells grown on 3-HPA and 4-HPA showed high HPC dioxygenase activities, whereas a lower activity was measured in glucose-grown cells (Fig. 4B). These results indicate that: i) key ring-cleavage dioxygenases of the homogenitase and the homoprotocatechuate pathways are functional, and ii) the homogenitase and the homoprotocatechuate central pathways are induced in strain LB400 during growth on 3-HPA and 4-HPA.

During growth of strain LB400 on 3-HPA, expression of the hmgA1 gene (BxeA2725) and hmgA2 gene (BxeA3900) were observed by RT-PCR. The hmgA1 gene is part of the hmgABC gene cluster in LB400, whereas the hmgA2 gene is clustered only with hmgB2 gene. In contrast, during 4-HPA degradation, the expression of hmgA2 gene but not of hmgA1 gene was observed. The results indicate that: i) the hmgABC gene cluster of strain LB400 is up regulated during 3-HPA metabolism but not during 4-HPA metabolism and, ii) two hmgA genes are transcribed during growth on 3-HPA. It is worth noting that these two genes have not identical DNA sequence. It is likely that one of the hmgA genes was acquired via horizontal gene transfer, which has been an important source of genes in strain LB400 [8]. These data indicates that growth on 3-HPA and 4-HPA are mediated by different cellular enzymatic components. During degradation of 3-HPA, it is likely that two hmgA genes copies are used, suggesting that enhanced catabolic abilities are required. Probably, the homogenitase pathway in strain LB400 is regulated at the level of HMG dioxygenase to prevent accumulation of catabolic intermediates that may have toxic effects on the cell. Expression of two gene copies encoding key enzymes may enhance catabolic machinery of strain LB400. The presence of multiples hmg gene copies spread in LB400 genome probably enhance its catabolic capabilities. The hmgAB arrangement, not linked with hmgC gene has also been observed in *Silicbacter pomeroyi*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Bordetella bronchiseptica* and *Cupriavidus necator* [7,19]. The use of the homogenitase pathway encoded by the hmgAB gene cluster has also proposed during growth on 3-HPA and 4-HPA in *C. necator* strain JMP134; in this strain the homoprotocatechuate pathway is absent [7]. The presence of multiples genes copies encoding chlorocatechol 1,2-dioxygenase in *C. necator* JMP134 are required for efficient chlorocatechol degradation during growth of strain JMP134 on 3-chlorobenzoate [38]. Enhanced degradation avoids accumulation of toxic metabolic intermediates such as catechols [5,39]. The toxicity of aromatic compounds and their metabolic intermediates for strain LB400 has been reported [9,31,35].

In addition to the expression of hmgA genes, the hpaD gene encoding HPC dioxygenase was expressed in LB400 during growth on 3-HPA and 4-HPA. A lower hpaD expression on glucose was observed. This in accordance with high HPC dioxygenase observed during growth in 3-HPA and 4-HPA and a lower activity observed in glucose-grown cells. In *Gammaproteobacteria*, 4-HPA is commonly degraded through the homoprotocatechuate pathway. 4-HPA degradation via HPC has been studied in some *E. coli* strains such as strain W that lacks the homogenitase pathway [2,18,29]. As mentioned above, the 4-HPA hydroxylase from *E. coli* strain W is able to hydroxylate a wide range of substrates, including 3-HPA [29]. Thus, we propose that the homoprotocatechuate pathway is an additional convergent route involved in 3-HPA and 4-HPA degradation in *B. xenovorans* LB400.

Proteomic analyses of LB400 cells grown on 3-HPA and 4-HPA provided additional evidence that the homogenitase central pathway is involved in the catabolism of 3-HPA and 4-HPA. The induction of fumarylacetoacetate hydratase HmgB by 3-HPA and 4-HPA was observed. The HmgB protein was induced in 3-HPA-grown cells (3.5-fold) and in 4-HPA-grown cells (2.5-fold).
compared to glucose-grown cells. As suggested above, 3-HPA hydroxylation mediated by the mhaAB gene products from strain LB400 is in accordance with 3-HPA catabolism via HMG revealed by proteomic analyses. 4-HPA degradation also has been reported to funnel into the homogentisate route, by hydroxylation at carbon 1 in Pseudomonas acidovorans and Azoarcus evansii [40,41]. Therefore, it is likely that 4-HPA is metabolized through the homogentisate pathway in LB400, in accordance with HmgB2 induction during 4-HPA metabolism observed by 2D-PAGE. The hmgB2 gene is adjacent to the hmgA2 gene (BxeA3900) in C1 of strain LB400. This is in accordance with hmgA2 expression observed during growth in 3-HPA and 4-HPA. The induction of HmgB protein suggests an up-regulation of the homogentisate pathway during 3-HPA and 4-HPA degradation in strain LB400. Moreover, HMG dioxygenase activity measured in crude extracts of strain LB400 suggested also induction of hmg catabolic genes. The HmgR regulator protein from P. putida strain U is an IclR-type regulator divergently transcribed from hmgABC cluster and acts as a repressor of hmg genes [19]. However, a gene similar to the HmgR protein from P. putida U was not identified in the hmgA1B1C1 cluster neighborhood and other regions of the genome of strain LB400. The transcription analyses suggested that expression of the homogentisate and homoprotocatechuate pathways are controlled at transcriptional level in LB400, in which the presence of specific substrates such as 3-HPA and 4-HPA are required. A gene encoding a HpaR regulator is located adjacent to the hpaGEDFHI gene cluster in LB400 genome. It has been determined that the HpaR protein of E. coli strain C regulates transcription of the hpaGEDFHI gene cluster and it is induced by 4-HPA or HPC [26]. It is likely that 4-HPA, 3-HPA or metabolic intermediates such as HPC may regulate expression of hpa cluster in LB400 mediated by HpaR (Fig. 1B).

Genome characterization of B. xenovorans LB400 revealed interesting catabolic capabilities. In this work, we provided evidence on the functionality of the two chromosomally encoded homogentisate
and homoprotoprotoocatechuate central pathways. In addition, it was shown that the 3-HPA and 4-HPA catabolic peripheral pathways are active. Moreover, two functional hmgA genes were used by LB400 during 3-HPA catabolism. Based on growth and degradation assays, gene expression analyses, protein synthesis, and key dioxygenases activity, we confirm that in strain LB400 3-HPA and 4-HPA separately funnel into both homogenetase and homoprotoprotoocatechuate central pathways. Probably, 3-HPA is preferentially channelled into the homogenetase central pathway, whereas 4-HPA is channelled actively in both central pathways. This study reveals the wide aromatic catabolic repertoire of strain LB400, linking the abilities predicted in silico with those observed at functional level. Furthermore, the utilization of both artho- and meta- cleavage pathways in the degradation of two isomers reflects the amazing metabolic plasticity of this bacterium.

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Author Contributions

Conceived and designed the experiments: VM LA MS. Performed the experiments: VM MG. Analyzed the data: VM LA MG MS. Contributed reagents/materials/analysis tools: MG MS. Wrote the paper: VM MS.