Genomic and Functional Analyses of the Gensitase and Protocatechuate Ring-Cleavage Pathways and Related 3-Hydroxybenzoate and 4-Hydroxybenzoate Peripheral Pathways in *Burkholderia xenovorans* LB400

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**Abstract**

In this study, the gentisate and protocatechuate pathways in *Burkholderia xenovorans* LB400 were analyzed by genomic and functional approaches, and their role in 3-hydroxybenzoate (3-HBA) and 4-hydroxybenzoate (4-HBA) degradation was proposed. The LB400 genome possesses two identical *mhbRTDH* gene clusters encoding the gentisate pathway and one *mhbM* gene encoding a 3-HBA 6-hydroxylase that converts 3-HBA into gentisate. The *pca* genes encoding the protocatechuate pathway and the *pobA* gene encoding the 4-HBA 3-monoxygenase that oxidizes 4-HBA into protocatechuate are arranged in gene clusters and single genes mainly at the minor chromosome, but also at the major chromosome and the megaplasmid. Strain LB400 was able to grow on gentisate, protocatechuate, 3-HBA and 4-HBA. Transcriptional analyses showed that the *mhbD* gene encoding the gentisate 1,2-dioxygenase was expressed during growth on 3-HBA, 4-HBA and gentisate, whereas the *pcaG* gene encoding the protocatechuate 3,4-dioxygenase was expressed only during growth on 4-HBA and protocatechuate. The *mhbM* gene encoding the 3,6-hydroxybenzoate was transcribed in strain LB400 during growth on HBAs, gentisate, protocatechuate and glucose. The *pobA* gene encoding the 4-HBA 3-monoxygenase was expressed during growth on HBAs and glucose. 3-HBA- and 4-HBA-grown LB400 cells showed gentisate 1,2-dioxygenase activity, whereas protocatechuate 3,4-dioxygenase activity was observed only in 4-HBA-grown cells. The *mhbRT* gene encoding a MarR-type transcriptional regulator that probably regulates the expression of the MhbT transporter, and the *pcaQ* and *pcaR* genes encoding LysR-type transcriptional regulators that regulate *pcaHG* and *pcaUBDC* genes, respectively, were transcribed during growth on both HBAs, gentisate, protocatechuate and glucose, suggesting a basal constitutive expression. The results indicate active gentisate, protocatechuate, 3-HBA and 4-HBA catabolic pathways in *B. xenovorans* LB400 and suggest that 3-HBA is channeled exclusively through the gentisate route, whereas 4-HBA is funneled into the protocatechuate central pathway and potentially into the gentisate pathway.

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**Introduction**

Aerobic bacterial degradation of aromatic compound proceeds generally in two phases. In the first phase, the aromatic compound is prepared for ring cleavage by a variety of ring modification reactions. The second phase of aerobic degradation includes ring fission of the aromatic compound and subsequent reactions leading to the formation of Krebs cycle intermediates [1–4]. *Betaproteobacteria* of the *Burkholderiales* order possess an amazing metabolic versatility to degrade a large number of aromatic compounds [1,3]. *Burkholderia xenovorans* LB400 is a bacterium able to degrade polychlorobiphenyls (PCBs) and diverse aromatic compounds [3–8]. LB400 genome has a size of 9.73 Mbp distributed in three replicons: the major chromosome (C1; 4.90 Mbp), the minor chromosome (C2; 3.36 Mbp) and the megaplasmid (MP; 1.47 Mbp) [3]. Genomic analysis of *B. xenovorans* strain LB400 revealed the presence of genes encoding an unusual high number of central and peripheral pathways for the degradation of aromatic compounds [3]. A recent study confirmed functionality of some predicted aromatic pathways [4]. However, the function of genes encoding diverse aromatic catabolic routes including the gentisate and protocatechuate pathways of *B. xenovorans* strain LB400 remains to be elucidated.

The gentisate pathway is a central route for the bacterial aerobic catabolism of benzoates such as 3-HBA and salicylate (2-hydroxybenzoate), phenolic compounds such as *m*-cresol and 2,5-xylene, and polycyclic aromatic hydrocarbons including naphthalene and phenanthrene [9–14]. Gentisate is cleaved by gentisate 1,2-dioxygenase (MhbD) to form maleylpyruvate. Maleylpyruvate is then isomerized by a glutathione-dependent
maleylpyruvate isomerase (MhbI) to yield fumarylpyruvate. A
fumarylpyruvate hydrodase (MhbH) catalyzes fumarylpyruvate
conversion into pyruvate and fumarate.

The gentisate catabolic pathway has been described as the
central route for 3-HBA degradation in some bacteria. 3-HBA is
degraded through the gentisate central pathway by the 3-HBA 6-
hydroxylase in Burkholderia cepacia J2315 [15], Pseudomonas alcaligenes
P2521 [9], Klebsiella pneumoniae M5a1 [11,16] and Salmonella
typhimurium [12]. The 3-HBA 6-hydroxylase is encoded by the
mhbM gene in K. pneumoniae M5a1 [11,16]. Alternatively, 3-HBA
could be degraded through the protocatechuate catabolic pathway
by the 3-HBA 4-hydroxylase, which is encoded by the mhbA gene in
Comamonas testosteroni K51 [17,18].

The protocatechuate pathway is a central catabolic route for
aromatic compounds, which is widely distributed among
taxonomically diverse bacteria and fungi [1–3,19,20]. Protocatechuate
is a key central intermediate in bacterial degradation of diverse
aromatic compounds, including 4-HBA, 3-HBA, vanillate, fer-
ulate, coniferyl alcohol, p-coumarate, phthalate, isophthalate and
terephthalate. Protocatechuate oxygenolytic ring-cleavage is
catalyzed by protocatechuate 3,4-dioxygenase (PcaGH) to generate
3-carboxy-cis,cis-muconate, which is converted into 4-carboxymu-
conolactone by 3-carboxy-cis,cis-muconate cycloisomerase (PcaB).
4-Carboxymuconolactone decarboxylase (PcaG) transforms the 4-
carboxymuconolactone into β-ketoadipate enol-lactone, which
is then hydrolyzed by β-ketoadipate enol-lactone hydrolase (PcaD)
into β-ketoacidipate. The enzyme β-ketoacidipate succinyl-CoA
transerase (PcaI) converts β-ketoacidipate into β-ketoacrylic-CoA,
which is finally transformed into succinyl-CoA and acetyl-CoA by
β-ketoacrylic-CoA thiolase (PcaF).

The protocatechuate central pathway is involved in 4-HBA
degradation in some microorganisms. 4-HBA is hydroxylated by
4-HBA 3-monooxygenase encoded by the polc gene to yield protocatechuate in Pseudomonas and Cupriavidus strains [2,21,22].

The aims of this study were to characterize the gentisate and
protocatechuate central pathways in B. xenovorans LB400 using
genomic and functional approaches. In addition, the role of these
ring-cleavage pathways during 3-HBA and 4-HBA degradation in
strain LB400 was studied. This report describes for the first time
the metabolism of 3-HBA and 4-HBA in B. xenovorans LB400. This study
demonstrates functional gentisate and protocatechuate catabolic pathways in B. xenovorans LB400 that are involved in the
degradation of 3-HBA and 4-HBA.

Materials and Methods

Chemicals
3,4-dihydroxybenzoic acid (protocatechuate, >98% purity),
2,5-dihydroxybenzoic acid (gentisate, 98% purity), 3-hydroxyben-
zoic acid (>99% purity) and 4-hydroxybenzoic acid (≥99% purity)
were obtained from Sigma-Aldrich (Saint Louis, MO,
USA).

Bacterial Strain and Culture Conditions
B. xenovorans strain LB400 was cultivated in M9 mineral medium
with trace solution and glucose (5 mM) [23], 3-HBA (5 and
10 mM), 4-HBA (5 mM), gentisate (5 mM) or protocatechuate
(5 mM) as sole carbon and energy source at 30 °C [3,23]. Growth
was determined by measuring turbidity at 525 nm and by
counting colony-forming units (CFU). Aliquots taken from
to bacterial cultures were diluted and plated on Luria-Bertani (LB)
medium. CFU mL−1 values were calculated as the mean ± SD of
values from at least three independent experiments.

Isolation of RNA and RT-PCR
RNA was isolated from LB400 cells grown until mid-exponential
growth phase (turbidity 0.525 nm 0.4–0.6) on 3-HBA, 4-HBA and
glucose. RNA was isolated using RNeasy mini kit (Qiagen, Hilden,
Germany). DNase I treatment was performed using the RNase-
Free DNase Set (Qiagen, Hilden, Germany). The RNA was
quantitated using a Qubit fluorometer (Invitrogen, Carlsbad, CA,
USA). In this study, specific primers for mhbB (BxeA2627,
BxeA4526), mhbT (BxeA2625, BxeA4528), mhbR (BxeA2624,
BxeA4529), mhbM (BxeB2526), pcaG (BxeB2776), pcaQ (BxeB2772),
pcaD (BxeB0642) and pobA (BxeA2040) genes were designed and
used (Table 1). Reverse transcription-PCR (RT-PCR) was carried out
with 40 μg of total RNA and the sequence-specific primers
using SuperScript One-step RT-PCR with Platinum Taq (Invitro-
gen, Carlsbad, CA, USA). Amplification of the 16S rRNA gene
was performed as control for DNA contamination using the
primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r
(5'-TACGCTATCTTTCGGATCGCA-3') as reported [4].
Negative and positive controls were included in each RT-PCR
assay. The expression of the constitutively expressed 16S rRNA
was used as a control to normalize across samples. At least three
independent RNA samples were collected at each condition and
two independent RT-PCR reactions for each sample were done to
assess reproducibility.

Preparation of Bacterial Cell Extracts
Cells grown until exponential phase with 3-HBA, 4-HBA or
glucose were harvested by centrifugation (10,733 g for 10 min) at
4 °C. Bacterial cells washed with sodium phosphate buffer
(50 mM, pH 7.0) and concentrated were disrupted using ultrasonic
cells disruptor Mircoson at 4 °C. Cell lysates were clarified by
centrifugation (19,300 g for 15 min) at 4 °C. The protease
inhibitor cocktail Tablet Complete mini (Roche, Indianapolis,
USA) was added to cell extracts to reach a final concentration of

| Table 1. Primer sets designed and used in this study. |
|-----------------------------------------------|
| **Gene** | **Name** | **Sequence (5’-3’)** | **Reference** |
|----------|---------|---------------------|--------------|
| mhbB1    | mhbDf   | 5'-ATCCGGGGGACCTTATTACCT-3' | This study   |
| mhbB2    | mhbDr   | 5'-GCCATTGCGATGCTGAAC-3' | This study   |
| mhbB1    | mhbRf   | 5'-GCAAGTGCCGCAATAATCCGTT-3' | This study   |
| mhbB2    | mhbRf   | 5'-TTGGCATGCGTAAAGCGCGGAT-3' | This study   |
| pcaGr    | pcaGf   | 5'-AAGAGCTTTCCCCTTTCTGCGCTG-3' | This study   |
| pcaG    | pcaGr   | 5'-CTCAAGAAACACGCCTTGCACA-3' | This study   |
| pcaQ    | pcaQf   | 5'-AAACTGTGACGGAGCGCGTTGAT-3' | This study   |
| pcaQ    | pcaQr   | 5'-AAAAAGGACCGGCGGACAGTGA-3' | This study   |
| pcaGr    | pcaRf   | 5'-ATGCACGAGCCAGAGCTGAAGT-3' | This study   |
| pcaGr    | pcaAr   | 5'-TGAACCTGGCATGACCGATTGCT-3' | This study   |
| mhbB    | mhbMf   | 5'-TACCGACCTGGAACAGCGGAGA-3' | This study   |
| mhbB    | mhbMr   | 5'-TATACGGACATACATGAGCCTG-3' | This study   |
| pobA    | pobAf   | 5'-TTGATCAGGGTCGTGCTGGAA-3' | This study   |
| pobA    | pobAr   | 5'-ACGCTATCTTGGCATGCGCA-3' | This study   |
| 165 rRNA | 27F     | 5'-AGAGTTTTGATCMTGGCTCAG-3' | [4,23]       |
| 165 rRNA | 1492R   | 5'-TAGGYYTACCTTGGACACTT-3' | [4,23]       |

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0.8 mg/ml. Protein concentration was measured using the Quick-it-T Protein Assay kit (Invitrogen, Carlsbad, CA, USA) that uses a fluorescent dye whose quantum yields are enhanced significantly when binding at the detergent-protein interface and measuring fluorescence at room temperature with the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) [24].

**Dioxygenase Activity Assays**

Gentisate 1,2-dioxygenase activity was determined spectrophotometrically by measuring the formation of maleylpyruvate at 330 nm [13]. The assay volume (10 ml) contained sodium phosphate buffer (50 mM, pH 7.0), crude extract (1000 µg of protein) and gentisate (0.6 mM). The reactions were carried out at 30°C and initiated by the addition of gentisate. Gentisate 1,2-dioxygenase activity was calculated using the molar extinction coefficient of maleylpyruvate 10,300 M⁻¹ cm⁻¹ [13,25]. Protocatechuic acid 3,4-dioxygenase activity was determined spectrophotometrically by the disappearance of protocatechuate measuring 330 nm [13]. The assay volume (10 ml) contained sodium phosphate buffer (50 mM, pH 7.0), crude extract (1000 µg of protein) and protocatechuate (0.6 mM). The reactions were carried out at 30°C and initiated by the addition of protocatechuate. Protocatechuic acid 3,4-dioxygenase activity was calculated using the difference of the molar extinction coefficient of protocatechuate and its product under these conditions that is 2,280 M⁻¹ cm⁻¹ [27]. One enzyme unit corresponds to the transformation under the conditions described above of 1 µmol of substrate to product per minute per milligram of protein at 30°C. The enzymatic activity assays were done in triplicate.

**Results**

**Genomic Analysis of the Gentisate Pathway and Related Peripheral Routes**

The mhb genes encoding the gentisate catabolic pathway were analyzed in the genome of *B. xenovorans* LB400. Table 2 describes the predicted genes encoding the gentisate central pathway and related peripheral routes in *B. xenovorans* LB400. Two identical mhbRRTDHI gene clusters encoding the gentisate pathway were identified at C1 (Fig. 1). Both mhb gene clusters are located adjacent in two identical chromosomal regions comprising thirty genes. Sequence analyses suggest that mhbD, mhbH and mhb genes of strain LB400 encode the gentisate 1,2-dioxygenase, maleylpyruvate isomerase and fumarylpyruvate hydratase, respectively. The protein sequences of strain LB400 encode the gentisate 1,2-dioxygenase, maleylpyruvate isomerase and fumarylpyruvate hydratase, respectively. The genes and genes encoding related peripheral pathways in *B. xenovorans* LB400 were distributed within the LB400 genome. Sequence analysis indicates that the mhbR regulator in *K. pneumoniae* M5a1 is a LysR-type activator of the mhbTgene cluster [39]. Genomic analyses indicated that two divergent σ²⁰-type promoters were present between mhbR and mhbT. A regulatory binding site with a palindromic sequence for a MarR-type transcriptional regulator (5’-ATTGTTTACA-CAAAACATAT-3’- ) was identified upstream from the mhbTTgene promoter, which is similar to the consensus MarR-type regulator binding site (5’-T/A/T/A/T/A/T/C/T-N₁₂-G/G/A/G/T/A/T/A/T-3’- ) [40].

Genome analysis indicated that an independent σ²⁰-type promoter is located upstream of the BxeA2626 (BxeA4527) and the mhbDHI genes. Upstream from the BxeA2626 (BxeA4527) gene promoter is located a LysR-type regulator binding sequence (5’-ATTCAGTTCAGAAT-3’- ) that has the conserved (5’-T-T-N₁₁-A-3’)- motif critical for binding of LysR-type transcriptional regulators [41]. The specific LysR-type transcriptional regulator gene of the mhbDHI gene cluster of strain LB400 has not been identified in this study.

Gene organization of the mhb cluster in *B. xenovorans* LB400 is similar to the mhb cluster from *K. pneumoniae* M5a1, except for the presence of a coding sequence of unknown function between mhbT and mhbD genes in strain LB400, the presence of the mhbM gene encoding 3-HBA 6-hydroxylase located downstream to mhbI in strain M5a1 and that the mhbR genes encode different transcriptional regulators (Fig. 3). The mhb gene clusters from other *Bacillus* strains including *Ralstonia solanacearum* GMI100, *Pseudomonas* CGD1, *P. naphthalenivorans* CJ2 and Acidovorax avenae subsp. avenae ATCC19860 showed different mhb gene organizations (Fig. 3).

A search of the gene encoding a 3-HBA 6-hydroxylase that funnel 3-HBA into the gentisate central pathway was performed in the LB400 genome. Sequence analysis indicates that the BxeB2526 gene (hereafter mhbM) located at C2 encodes a 3-HBA 6-hydroxylase (Fig. 1). The mhbM gene product from strain LB400 shared a 38% sequence identity with 3-HBA 6-hydroxylase (MhbM) from *K. pneumoniae* M5a1 [30]. A 4-HBA 1-hydroxylase activity that converts 4-HBA into gentisate has been described [42]. However, the gene sequence encoding 4-HBA 1-hydroxylase has not been reported yet, impeding a further genomic analysis.

**Genomic Analysis of Protocatechuate Pathway and Related Peripheral Routes**

Predicted pca genes encoding the protocatechuate catabolic pathway are distributed within the *B. xenovorans* LB400 genome (Fig. 4). Predicted pca genes encoding the protocatechuate pathway and related peripheral routes are listed in Table 2. Sequence analyses suggest that pcaG, pcaH, pcaQ, pcaF (BxeB2167; hereafter pcaF1), pcaC, pcaD (BxeB0646; hereafter pcaD1), pcaB (BxeB0645; hereafter pcaB1), pcaJ (BxeB0644; hereafter pcaJ1), pcaI (BxeB0643; hereafter pcaI1), pcaQ and pcaR genes encoding the protocatechuate catabolic pathway are located at C2. Copies of pcaB (BxeB1906; hereafter pcaB2) and pcaD genes (BxeB0595; hereafter pcaD2) were identified at C2. Two pcaF genes (hereafter pcaF2 and pcaF3, respectively) and a pcaJ gene copy (hereafter pcaJ3) were identified by sequence analyses at C1. In addition, pcaQ and pcaI genes copies (hereafter pcaQ2 and pcaI2, respectively) were identified by sequence analyses at MP. The pcaG and pcaH genes encoding the alpha and beta subunits from a protocatechuate 3,4-dioxygenase were located 2.3 Mbp distant region from pcaF1BD gene cluster. The pcaG and pcaH gene products of strain LB400 showed ≥90% identity with the PcaG and PcaH proteins from *Acinetobacter lwoffi* K24 [31]. The spread in the organization of pca genes and genes encoding related peripheral pathways in *B. xenovorans* LB400 is also observed in *Cupriavidus metallidurans* CH34.
Figure 1. Predicted mhb genes encoding the gentisate central pathway and related genes in B. xenovorans LB400. The mhb genes located at the major chromosome (C1) and the minor chromosome (C2). The genes encoding gentisate 1,2-dioxygenase are indicated with black arrows. The orientations of ORFs are represented by open arrows. The promoter regions for mhbR1, mhbR2, mhbT1, mhbT2, BxeA2626 and BxeA4527 are denoted with small black arrows, bent in the directions of transcription. The sizes of the genes and the intergenic regions are on scale.
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Figure 2. Relationship between bacterial transcriptional regulators for gentisate and protocatechuate catabolic pathways. LysR, MarR and IclR transcriptional regulators from B. xenovorans LB400 and other bacteria were depicted. The dendogram was obtained by the neighbor-joining method using MEGA 5.0 based on sequence alignment calculated by CLUSTAL W using the default options. Circles denote proteins encoded in the genome from B. xenovorans LB400. The sequence and their accession number are: CatM, Acinetobacter baylyi ADP1 (P07774); BenM, A. baylyi ADP1 (AAC46441); TfgT, Cupriavidus pinatubonensis JMP134 (AAC44724); CdoR, Comamonas sp. JS765 (AAC79916); MhbR, Klebsiella pneumoniae M5a1 (Q5EXK6); PcaQ, Agrobacterium tumefaciens C58/ATCC3397 (P0A4T6); PcaQ, B. xenovorans LB400 (Q13RR8); SalR, A. baylyi ADP1 (AAF04311); NagR, Ralstonia sp. U2 (Q9EXL7); PcaR, B. xenovorans LB400 (ABE35311); MhbR, B. xenovorans LB400 (ABE30329); BadR, Rhodopseudomonas palustris CGA009 (AACC23923); CbaR, Comamonas testosteroni TA441 (Q9EXL7); HpaR, Escherichia coli W (Z37980); MhpR, C. testosteroni TA441 (P77569); PcaR, Pseudomonas putida PRS2000 (Q52154); CatR, Rhodococcus opacus (Q33539); PcaR, P. putida KT2440 (Q8RN41); PcaR, A. tumefaciens C58 (Q7CV82); PcaU, A. baylyi ADP1 (AAC37157); PobR, A. baylyi ADP1 (A36893).
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Table 2. Predicted genes encoding the gentisate and protocatechuate pathways and peripheral reactions from B. xenovorans LB400.

| Gene* | ORF | aa | Related gene products |
|-------|-----|----|-----------------------|
| mhbR1 mhbR2 | BxeA2624 BxeA4529 | 166 MhbR | Transcriptional regulator, MarR family |
| mhbT1 mhbT2 | BxeA2625 BxeA4528 | 466 PcaK | 4-Hydroxybenzoate transporter |
| mhbD1 mhbD2 | BxeA2627 BxeA4526 | 348 MhbD | GentiKate-1,2-dioxygenase |
| mhbH1 mhbH2 | BxeA2628 BxeA4525 | 232 MhbH | Fumarlypyruvate hydrolase |
| mhbI3 mhbI2 | BxeA2629 BxeA4524 | 216 MhlI | Maleylpyruvate isomerase |
| pcaG | BxeB2776 | 195 PcaG | Protocatechuate 3,4-dioxygenase, alpha subunit |
| pcaI | BxeB2775 | 234 PcaI | Protocatechuate 3,4-dioxygenase, beta subunit |
| pcaQ | BxeB2772 | 310 PcaQ | Transcriptional regulator, LysR family |
| pcaF1 | BxeB2157 | 400 PcaF | β-Ketoadipate:CoA transferase, alpha |
| pcaF2 | BxeA0469 | 400 PcaF | β-Ketoadipate:CoA transferase, alpha |
| pcaF | BxeA4255 | 402 PcaF | β-Ketoadipate:CoA transferase, alpha |
| pcaC | BxeB0647 | 130 PcaC | 4-Carboxymuconolactone decarboxylase |
| pcaD1 | BxeB0646 | 263 PcaD | β-Ketoadipate:Enol-lactone hydrolase |
| pcaD2 | BxeB0655 | 265 PcaD | β-Ketoadipate:Enol-lactone hydrolase |
| pcaR1 | BxeB0645 | 459 PcaR | 3-Carboxylic acid muconic acid cycloisomerase |
| pcaB2 | BxeB0646 | 445 PcaB | 3-Carboxylic acid muconic acid cycloisomerase |
| pcaR1 | BxeB0644 | 219 PcaR | β-Ketoadipate-succinyl-CoA transferase, beta subunit |
| pcaI1 | BxeC0572 | 265 PcaI | β-Ketoadipate-succinyl-CoA transferase, beta subunit |
| pcaO3 | BxeA1366 | 234 PcaO | β-Ketoadipate-succinyl-CoA transferase, beta subunit |
| pcaF1 | BxeB0643 | 233 PcaF | β-Ketoadipate-succinyl-CoA transferase, alpha subunit |
| pcaO2 | BxeC0572 | 235 PcaO | β-Ketoadipate-succinyl-CoA transferase, alpha subunit |
| pcaR | BxeB0642 | 309 PcaR | Transcriptional regulator, LysR family |
| pobA | BxeA2040 | 394 PobA | 4-Hydroxybenzoate-3-monoxygenase |

*Genes for those cases where biochemical or genetic evidence for function is available are underlined.

(Fig. 5). Predicted pcaQ gene located next to the pcaHG genes and divergently transcribed encodes a LysR-type transcriptional regulator (Fig. 2) that might regulate the expression of the pcaHG gene cluster. The PcaQ protein shared a 48% sequence identity with the LysR-type transcriptional regulator PcaQ from Agrobacterium tumefaciens A348 (Table 2) [43]. Two divergent σ70-type promoters are present between the pcaQ gene and the pcaGH gene cluster. A PcaQ LysR-type regulatory binding sequence (5′-T-N11-A-3′) was identified upstream of the pcaI gene promoter. This regulatory sequence has the consensus sequence for the PcaQ regulator recognition site sequence of Burkholderia megaterium (5′-ATAACGCCAGTTAT-3′) [43]. Predicted pcaR gene (BxeB0642) located adjacent to the pcaIJBD gene and divergently transcribed encodes a LysR-type transcriptional regulator (Fig. 2). The pcaR gene product shared a 95% sequence identity with a LysR-type transcriptional regulator adjacent to the pcaIJBD gene cluster from B. phytofirmans PsJN (Table 2) [38]. Genomic analysis of the pcaCD1B1J1I1R gene cluster showed that two divergent σ70-type promoters are present between the pcaR gene and the pcaCD1B1J1I1 gene cluster. A putative LysR-type regulatory binding sequence (5′-TGGCGCTAAACG-3′) was identified upstream of the pcaR gene promoter. This sequence has the conserved (5′-T-N17-A-3′) motif critical for binding of LysR-type autoregulatory transcriptional regulators [41].

The genes encoding the enzymes of the 3-HBA and 4-HBA peripheral reactions that funnel into the protocatechuate pathway were searched in the LB400 genome. The pobA gene (BxeA2040)
encoding for a 4-HBA 3-monoxygenase that converts 4-HBA into protocatechuate was identified by sequence analyses at C1 (Fig. 4). The \( \text{pohA} \) gene protein from strain LB400 shares 51% sequence identity with the PohA enzyme from \( P. \) fluorescens strain Pf-5 [21]. In addition, protocatechuate has been described as a central route for 3-HBA degradation by a 3-HBA 4-hydroxylase encoded by the \( \text{mobA} \) gene in bacteria. The \( \text{mobA} \) gene was not found in the LB400 genome.

**Growth of Strain LB400 on Gentisate, Protocatechuate and HBAs**

Sequence analyses of \( B. \) xenovorans LB400 genome revealed the presence of genes encoding the gentisate and the protocatechuate ring-cleavage pathways and 3-HBA and 4-HBA peripheral pathways. To investigate whether these catabolic pathways are functional, in a first approach growth assays were performed. The growth of \( B. \) xenovorans strain LB400 on the central metabolites gentisate and protocatechuate was studied. \( B. \) xenovorans LB400 was able to grow on gentisate (5 mM) and on protocatechuate (5 mM) as sole carbon and energy source (Fig. 6). In addition, the growth of \( B. \) xenovorans strain LB400 on 3-HBA or 4-HBA was studied. Strain LB400 was able to grow on 3-HBA (10 mM) and 4-HBA (5 mM) (Fig. 6). \( B. \) xenovorans LB400 attained higher cell concentration at stationary phase on protocatechuate (1.1 \( \times 10^9 \text{ CFU mL}^{-1} \)) and 4-HBA (1.0 \( \times 10^9 \text{ CFU mL}^{-1} \)). The growth of strain LB400 on gentisate or 3-HBA yielded 7.0 \( \times 10^8 \text{ CFU mL}^{-1} \) (Fig. 6). These results indicate functional 3-HBA and 4-HBA peripheral pathways and gentisate and protocatechuate central pathways in \( B. \) xenovorans LB400.

**Transcriptional Analyses of the \( \text{mhb} \) and \( \text{pca} \) Genes**

In order to determine the role of predicted genes encoding the gentisate and protocatechuate ring-cleavage routes and 3-HBA and 4-HBA peripheral pathways in strain LB400, transcriptional analyses of key catabolic genes were performed. In addition, the expression of genes encoding potential transcriptional regulators was analyzed. The expression of the \( \text{mhbD} \) gene encoding the enzyme gentisate 1,2-dioxygenase during exponential growth phase of LB400 cells grown on gentisate, protocatechuate, 3-HBA, 4-HBA, and glucose was analyzed. The \( \text{mhbD} \) gene was transcribed during growth of strain LB400 in gentisate, 3-HBA and 4-HBA (Fig. 7). In contrast, expression of the \( \text{mhbD} \) gene was not observed during growth on protocatechuate or glucose (Fig. 7). The \( \text{mhbR} \) gene encoding a MarR-type transcriptional regulator was transcribed during LB400 growth on 3-HBA, 4-HBA, gentisate, protocatechuate and glucose (Fig. 7). These results suggest that gentisate, 3-HBA and 4-HBA or its metabolites induce transcription of the \( \text{mhbD} \) gene in \( B. \) xenovorans LB400. On the other side, the \( \text{mhbR} \) gene has probably a basal constitutive expression. Transcription analysis of the \( \text{mhbM} \) gene encoding the 3-HBA 6-hydroxylase was performed. The \( \text{mhbM} \) gene of strain LB400 was expressed during growth on 3-HBA, 4-HBA, gentisate, protocatechuate and glucose (Fig. 7). However, higher
expression of the mhbM gene on HBAs than on glucose was observed.

Transcription of the pcaG gene encoding the key protocatechu- 
ate 3,4-dioxygenase alpha subunit during growth of strain LB400 
with different aromatic substrates was studied. Predicted pcaG gene 
was expressed during growth of strain LB400 on protocatechu- 
ate and 4-HBA (Fig. 7). However, pcaG transcripts were not observed 
during growth of strain LB400 on gentisate, 3-HBA and glucose. 
These results suggest that by 4-HBA, protocatechuate or its 
metabolites induce the transcription of the pcaG gene. Both pcaQ 
and pcaR genes encoding the transcriptional regulators of the 
pcaGH genes and the pcaIJBDC gene cluster were transcribed 
during LB400 growth on HBAs, gentisate, protocatechuate and 
glucose (Fig. 7). Therefore, the pcaQ and pcaR genes have probably 
a basal constitutive expression.

Transcription of the pobA gene encoding the 4-HBA 3-
monooxygenase of strain LB400 was analyzed. The pobA gene 
was expressed during LB400 growth only on 3-HBA, 4-HBA and 
glucose (Fig. 7). The pobA gene expression was higher during 
growth on HBAs than on glucose.

These results indicate that expression of the mhb and pca genes 
encoding catabolic enzymes is regulated in LB400 cells. These 
regulations are probably exerted by MarR-type and LysR-type 
transcriptional regulators. This study also suggests a regulated 
expression of the mhbM gene encoding the peripheral 3-HBA 6-
hydroxylase and of the pobA gene encoding the peripheral 4-HBA 
3-monooxygenase but with a basal constitutive expression of the 
mhbM gene. Overall, the transcriptional analyses suggest that 3-
HBA is catabolized and funneled exclusively through the gentisate 
central pathway, whereas 4-HBA can be degraded through the 
protocatechuate and gentisate central pathways in strain LB400.

Gentisate and Protocatechuate Ring-cleavage Activities

To further characterize the gentisate and protocatechuate 
central pathways in B. xenovorans strain LB400, the activities 
of the key enzymes gentisate 1,2-dioxygenase and protocatechuate 
3,4-dioxygenase were measured. Both dioxygenase activities were 
determined in crude extract of cells cultured until mid exponential 
phase in 3-HBA, 4-HBA and glucose. Higher gentisate 1,2-
dioxygenase activity was observed in cells grown on 3-HBA

Figure 4. Genetic organization of the pca genes involved in protocatechuate catabolism in Burkholderia xenovorans LB400. The pca 
genes are located in the minor chromosome (C2), major chromosome (C1) and megaplasmid (MP). The genes encoding the alpha and beta subunits 
of the protocatechuate 3,4-dioxygenase are indicated with black arrows. The orientations of ORFs are represented by open arrows. The promoter 
regions for pcaH, pcaQ, pcaI and pcaR are denoted with small black arrows, bent in the directions of transcription. The sizes of the genes and 
the intergenic regions are on scale.

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(0.053±0.001 U mg\(^{-1}\) protein) and 4-HBA (0.049±0.004 U mg\(^{-1}\) protein), whereas the gentisate 1,2-dioxygenase activity was not detected in glucose-growth cells (Fig. 8A). LB400 cells grown on 4-HBA showed high protocatechuate 3,4-dioxygenase activity (0.15±0.02 U mg\(^{-1}\) protein), whereas the protocatechuate dioxygenase activity was not observed in LB400 cells grown on 3-HBA and glucose (Fig. 8B). Enzyme activity assays suggest an active protocatechuate pathway in 4-HBA-grown cells, whereas the gentisate central pathway was found to be active in both 3-HBA- and 4-HBA-grown cells.

**Discussion**

This study has focused on a genomic analysis on the gentisate and protocatechuate catabolic pathways and its related peripheral pathways in *B. xenovorans* LB400. Experimental evidence was provided on the functionality of these ring-cleavage pathways, and their roles during 3-HBA and 4-HBA catabolism were proposed. Strain LB400 is able to grow on gentisate as sole carbon source, indicating a functional gentisate catabolic pathway. The gentisate catabolic pathway is present only in few *Burkholderia* strains [1]. Both *mhbRTDHI* gene clusters were found in two identical and adjacently located segments of 30 genes in LB400 major chromosome, indicating a gene cluster duplication process. Gene duplication is frequently observed in aromatic catabolic pathways and suggests functional redundancies, which could potentially increase the bacterial fitness and its adaptation to adverse environmental conditions. The *mhbDHI* gene cluster organization...
strains from Burkholderiales order including *B. xenovorans* LB400. However, the complete mhbRTDHI gene cluster with a coding sequence of unknown function between mhbT and mhbD was found in strain LB400, which has not been observed in other bacteria and its function remains to be elucidated.

Growth studies indicated that a 3-HBA peripheral pathway is present in *B. xenovorans* LB400. Gentisate has been described as the main ring-cleavage pathway for 3-HBA assimilation in bacteria, such as *K. pneumoniae* M5a1 [11], *Corynebacterium glutamicum* RES167 [44] and *Rhodococcus* sp. strain NCIMB 12038 [45]. In *B. xenovorans* strain LB400 the mhbM gene encoding the 3-HBA 6-monoxygenase is located at C2, whereas the mhbRTDHI gene cluster is located at C1 (Fig. 1). In contrast, in strains M5a1, RES167, NCIMB 12038 and in *Comamonas* and other *Burkholderia* strains, a 3-HBA 6-hydroxylase-encoding mhbM gene is usually found within the mhbDHI gene cluster. An increased transcription of mhbM gene

Figure 7. Transcriptional analysis of the mhb and pca genes during LB400 growth on hydroxybenzoates, gentisate and protocatechuate. LB400 cells were grown on 3-HBA (lane 1), 4-HBA (lane 2), gentisate (lane 3), protocatechuate (lane 4) and glucose (lane 5) as sole carbon source. (A) Transcription of mhbD and mhbR genes; (B) Transcription of pcaG, pcaQ and pcaR genes. (C) Transcription of mhbM and pobA genes. RT-PCR (15, 20 and 25 amplification cycles) assays were performed using RNA extracted from LB400 cells collected at mid exponential growth phase. Transcription of 16S rRNA gene was used as control (not shown).

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Figure 8. Gentisate and protocatechuate dioxygenase activities of *B. xenovorans* LB400. Cells were grown on 3-HBA (black square), 4-HBA (empty square) and glucose (empty circles) as sole carbon source. (A) Gentisate 1,2-dioxygenase activity measured by maleylpyruvate formation. (B) Protocatechuate 3,4-dioxygenase activity measured by protocatechuate disappearance. Each point is an average ± SDs of values from three independent assays.

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in strain LB400 was observed during growth on 3-HBA. Therefore, we propose that the predicted mhbM gene product of strain LB400 is involved in the hydroxylation of 3-HBA to gentisate.

It is worth noting that B. xenovorans LB400 was able to grow using 10 mM 3-HBA, whereas no growth of LB400 cells was achieved on 5 mM 3-HBA. We propose that a 3-HBA (5 mM) did not support the growth of B. xenovorans strain LB400, probably due to an insufficient transport of 3-HBA across the membrane. These results suggest that strain LB400 lacks an active transport for 3-HBA. 4-HBA catabolism through the gentisate pathway has been described in bacterial strains from 

Betaproteobacteria genus [46] and the "archaical isolate Halobacula sp. strain D1" [42]. B. xenovorans LB400 was able to grow using 4-HBA (5 mM) as sole carbon source. The LB400 growth on 4-HBA (5 mM) but not on 3-HBA (5 mM) suggests an efficient transport of 4-HBA rather than 3-HBA through the membrane by an active 4-HBA transport system. This is supported by our bioinformatics analyses that indicated that the mhbT gene product of strain LB400 has 33% sequence identity with the PcaK protein of P. putida PRS2000, which is an active transporter system for 4-HBA [29,33]. The mhbT gene of B. xenovorans LB400 encodes a MFS transporter of the AAH5 family that may be involved in the active transport of gentisate and 4-HBA.

Genomic analyses suggest that the mhbR gene, which is upstream and divergently transcribed to the mhbT gene, encodes for a transcriptional regulator of the mhbT gene in strain LB400. The mhbR gene of strain LB400 was transcribed during growth on 3-HBA, 4-HBA, gentisate, protocatechuate and glucose, suggesting a basal constitutive expression. The mhbR gene product from B. xenovorans LB400 shares 30% sequence identity with MarR-type transcriptional regulators from the mhb gene cluster from P. naphthalenivorans CJ2 [28] and C. testosteroni CNB-2 [47]. MarR regulators are winged helix-turn-helix DNA-binding proteins that respond to specific ligands and act as dimmers. In absence of the ligand, MarR regulators bind to palindromic sequences within the promoter, generally causing transcriptional repression [40]. We propose that the Mar-R-type regulator encoded by the mhbR gene from B. xenovorans LB400 is a transcriptional repressor that regulates the expression of the mhbT gene. Transcriptional analysis showed that the mhbD gene encoding a gentisate 1,2-dioxygenase was expressed during growth on gentisate, 3-HBA and 4-HBA, but not on glucose. Transcriptional analyses suggest that either 3-HBA, 4-HBA, gentisate or its metabolic intermediates induced the transcription of the mhb gene cluster. The expression of mhbD gene correlates with an increased gentisate 1,2-dioxygenase activity observed in LB400 cells grown on 3-HBA and 4-HBA. Genomics analysis showed the presence of a conserved sequence (5'-T-N11-T-A-3') critical for LysR-type transcriptional regulator binding, located upstream of the -35 box of BxcA2626 and BxcA4527 genes. However, a LysR-type transcriptional regulator gene was not found in the neighborhoods of both mhb gene clusters. Although further studies are needed to survey the regulation mechanisms of 3-HBA and 4-HBA degradation pathways via gentisate in strain LB400, these genomic analyses suggest that mhbDIII cluster is regulated by a LysR-type transcriptional regulator in strain LB400. Overall, transcriptomic analyses and dioxygenase activity assays indicate that the gentisate pathway in strain LB400 is active during 3-HBA and 4-HBA degradation, suggesting that both substrates may be funneled into the gentisate central pathway (Fig. 9). We propose that a 4-HBA 1-hydroxylase could be involved in the conversion of 4-HBA into gentisate in B. xenovorans LB400. Experimental data with deuterated substrates in the archaical strain Halobacula sp. D1 supported the mechanism in which 4-HBA is converted into gentisate by hydroxylation of 4-HBA at C-1, with a concomitant 1,2-carboxyl group migration. The transformation is called NIH shift and is catalysed by a 4-HBA 1-hydroxylase [42]. Although no gene sequence information of the 4-HBA 1-hydroxylase enzyme is available to perform a genomic search, an active gentisate pathway during 4-HBA assimilation suggests a 4-HBA 1-hydroxylase activity in B. xenovorans strain LB400. Recently, we reported that B. xenovorans strain LB400 degrades 4-hydroxyphenylacetate via homogentisate, in which also a migration of the acetate group by a NIH shift reaction may also be involved [4]. Hydroxylation of monosubstituted aromatic rings at C-1, followed by a migration of the carboxymethyl side chain to C-2 has been described in P. acidovorans and Nitrosomonas europaea 19718 [48,49]. Future studies are needed to characterize the genes encoding the 4-HBA 1-hydroxylase that converts 4-HBA into gentisate in bacteria, including B. xenovorans strain LB400.

Most bacteria metabolize 4-HBA by hydroxylation at C-3 to yield protocatechuate as central intermediate [1,2]. The 4-HBA 3-monooxygenase encoded by the pobA gene catalyzes 4-HBA conversion into protocatechuate in Pseudomonas strains [21,22]. Genomic analyses revealed the presence of the pca genes encoding the complete protocatechuate central pathway are located at C2 and pca gene copies are located at C1 and MP, while the pobA gene is separately located at C1 in strain LB400 genome. Spreading of pca genes and genes encoding its related peripheral reactions is also observed in Betaproteobacteria, such as Cupriavidus (Fig. 4), Burkholderia and Ralstonia strains [1]. Diverse pca gene organizations have been reported in bacteria [1,2,20]. The pcaGH genes are clustered with other pca genes of the protocatechuate route and related peripheral pathways in the Gamma proteobacteria A. baylyi ADP1 and P. putida KT2440, the Alphaproteobacteria strains Agrobacterium tabaci and Sinorhizobium meliloti 1021, and the Betaproteobacteria from the Cupriavidus genus such as C. metallidurans CH34, C. pinabutonensis JPMP13 and C. necator H16 [1]. In contrast, in B. xenovorans LB400 and other bacterial strains from the Burkholderia genus, the pcaGH genes are not clustered with other pca genes. B. xenovorans strain LB400 possesses the pcaQHG and the pcaRTBDC gene clusters and separately pcaF gene copies. In addition, LB400 genome contains a pcaT gene cluster and pcaB, pcaD and pcaF gene single-copies, indicating genetic redundancy.

B. xenovorans LB400 was able to grow on 4-HBA and protocatechuate, indicating functional 4-HBA peripheral and protocatechuate ring-cleavage pathways. Transcriptional analyses showed that pcaG gene encoding the protocatechuate 3,4-dioxygenase alpha subunit was expressed during protocatechuate and 4-HBA degradation by strain LB400. The expression of the pcaG gene during 4-HBA degradation correlates with an increased protocatechuate 3,4-dioxygenase activity observed in LB400 cells extracts grown on 4-HBA. In contrast, neither pcaG gene expression nor protocatechuate 3,4-dioxygenase activity was observed in 3-HBA and glucose-grown LB400 cells. These results suggest that 4-HBA is exclusively degraded via protocatechuate, in which a hydroxylation of 4-HBA at C-3 occurred for protocatechuate formation. The pobA gene of strain LB400 was transcribed during growth on 4-HBA, suggesting that the pobA gene product is involved in 4-HBA hydroxylation to produce protocatechuate in B. xenovorans strain LB400 (Fig. 9). B. xenovorans LB400 as well as most strains belonging to Burkholderia and Ralstonia genera that possess the pcaGH genes have a 4-HBA 3-hydroxylase-encoding gene, indicating that protocatechuate is the preferred catabolic pathway for 4-HBA degradation. In addition, these results suggest that either 4-HBA, protocatechuate or its metabolic intermediates induced the transcription of protocatech-
The pcaQ gene adjacent to pcaGH genes encodes a LysR-type transcriptional regulator and was transcribed during growth on 4-HBA, protocatechuate, 3-HBA, gentisate and glucose, suggesting a constitutive expression of the pcaQ gene. Thus, the PcaQ LysR-type transcriptional regulator of strain LB400 probably controls the expression of the pcaGH genes during 4-HBA and protocatechuate degradation in strain LB400. LysR-type transcriptional regulators act as activators during aromatic compounds catabolism. In general, the inducers are metabolic intermediates of the catabolic pathway. The enzymes are: PobA (4-hydroxybenzoate 3-monooxygenase), PcaGH (protocatechuate 3,4-dioxygenase), PcaB (3-carboxymuconate cycloisomerase), PcaC (4-carboxymuconolactone decarboxylase), PcaD (β-ketoacidipate enol-lactone hydrolase), PcaJ (β-ketoacidipate:succinyl-CoA transferase) and PcaF (β-ketoacidipyl-CoA thiolase).

Enzymes for the degradation of 3-carboxy-cis,cis-muconate into Krebs cycle intermediates [2,20] were observed in the closely related strain B. phytofirmans PsJN. In accordance with this, it has been reported that all strains of the ‘B. cepacia complex’ as well as B. phymatum STM815 and B. glumae BGR1 possess a LysR-type transcriptional regulator gene clustered with the pcaIJBDC genes [1]. It is likely that the pcaR gene product of strain LB400 acts as a transcriptional activator of the expression of the pcaIJBD gene cluster. Further studies are required to confirm that PcaQ and PcaR proteins regulate the expression of the pcaIJBDC gene clusters in B. xenovorans LB400. The protocatechuate pathway is widespread among pathogenic and environmental bacteria, and only a small number of strains lack this pathway. Therefore, the protocatechuate catabolic pathway can be an important catabolic feature and play a key role to improve bacterial fitness in the environment and in pathogenesis.

Figure 9. Models of 3-HBA and 4-HBA catabolic pathways in B. xenovorans LB400. (A) 3-HBA and 4-HBA catabolism via the gentisate central pathway (continuous line). The substrates and products are: 3-hydroxybenzoate (1); gentisate (2); maleylpyruvate (3); fumarylpyruvate (4); pyruvate (5); fumarate (6) and 4-hydroxybenzoate (7). The enzymes are MhbM (3-hydroxybenzoate hydrolase), MhbD (gentisate 1,2-dioxygenase), MhbH (fumarylpyruvate hydrolase). The gene encoding the 4-HBA 1-hydroxylase is unknown. (B) 4-HBA catabolism via protocatechuate (dotted line). The substrates and products are: 4-hydroxybenzoate (7); protocatechuate (8); 3-carboxy-cis,cis-muconate (9); 4-carboxymuconolactone (10); β-ketoacidipate enol-lactone (11); β-ketoacidipate (12); β-ketoacidipyl-CoA (13); Acetyl-CoA (14); Succinyl-CoA (15). The enzymes are: PobA (4-hydroxybenzoate 3-monooxygenase), PcaGH (protocatechuate 3,4-dioxygenase), PcaB (3-carboxymuconate cycloisomerase), PcaC (4-carboxymuconolactone decarboxylase), PcaD (β-ketoacidipate enol-lactone hydrolase), PcaJ (β-ketoacidipate:succinyl-CoA transferase) and PcaF (β-ketoacidipyl-CoA thiolase). doi:10.1371/journal.pone.0056038.g009
In this report, we described functional genitase and protocatechuate ring-cleavage pathways in B. xenovorans LB400, revealing inducible expression of key genes encoding genitase and protocatechuate dioxygenases. Additionally, in this study 3-HBA and 4-HBA peripheral reactions in strain LB400 were reported. Based on genotypic and biochemical analyses, geno-expression analyses and key dioxygenase activities, we propose that 3-HBA degradation is channeled exclusively through the genitase central pathway in B. xenovorans strain LB400, whereas 4-HBA can be degraded and funneled actively into the protocatechuate and genitase central pathways (Fig. 9). This report reveals novel catabolic capabilities of B. xenovorans LB400, which can use two different ring-cleavage pathways for the metabolism of 3-HBA and 4-HBA. This study confirms that B. xenovorans LB400 possesses a high metabolic versatility, which is reflected on its extensive gene and functional redundancy for the degradation of aromatic compounds.

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Author Contributions

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

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