Genomic and Functional Analyses of the 2-Aminophenol Catabolic Pathway and Partial Conversion of Its Substrate into Picolinic Acid in *Burkholderia xenovorans* LB400

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**Abstract**

2-aminophenol (2-AP) is a toxic nitrogen-containing aromatic pollutant. *Burkholderia xenovorans* LB400 possess an *amn* gene cluster that encodes the 2-AP catabolic pathway. In this report, the functionality of the 2-aminophenol pathway of *B. xenovorans* strain LB400 was analyzed. The *amnRJBACDFEHG* cluster located at chromosome 1 encodes the enzymes for the degradation of 2-aminophenol. The absence of *habA* and *habB* genes in LB400 genome correlates with its no growth on nitrobenzene. RT-PCR analyses in strain LB400 showed the co-expression of *amnB*, *amnBAC*, *amnACD*, *amnDFE* and *amnEHG* genes, suggesting that the *amn* cluster is an operon. RT-qPCR showed that the *amnB* gene expression was highly induced by 2-AP, whereas a basal constitutive expression was observed in glucose, indicating that these *amn* genes are regulated. We propose that the predicted MarR-type transcriptional regulator encoded by the *amnr* gene acts as repressor of the *amn* gene cluster using a MarR-type regulatory binding sequence. This report showed that LB400 resting cells degrade completely 2-AP. The *amn* gene cluster from strain LB400 is highly identical to the *amn* gene cluster from *P. knackmussi* strain B13, which could not grow on 2-AP. However, we demonstrate that *B. xenovorans* LB400 is able to grow using 2-AP as sole nitrogen source and glucose as sole carbon source. An *amnBA* mutant of strain LB400 was unable to grow with 2-AP as nitrogen source and glucose as carbon source and to degrade 2-AP. This study showed that during LB400 growth on 2-AP this substrate was partially converted into picolinic acid (PA), a well-known antibiotic. The addition of PA at lag or mid-exponential phase inhibited LB400 growth. The MIC of PA for strain LB400 is 2 mM. Overall, these results demonstrate that *B. xenovorans* strain LB400 posses a functional 2-AP catabolic central pathway, which could lead to the production of picolinic acid.

**Introduction**

Several aromatic compounds with nitro and amino groups such as 2-aminophenol (2-AP) are toxic and persistent organic pollutants (POPs) in the environment. 2-AP is a compound used in the production of dyes, plastics and pharmaceuticals, which could be released in industrial wastewaters, polluting the environment [2, 3]. 2-AP is cytotoxic [3] and has carcinogenic potential [4]. Therefore, the removal of 2-AP is required for the clean-up of polluted sites. Some microorganisms are able to degrade aerobically 2-AP [1, 5].

The aerobic 2-AP metabolic pathway (Fig. 1) has been described in *Pseudomonas pseudoalcaligenes* JS45, *Pseudomonas putida* HS12, *Pseudomonas sp.* AP-3 and *Pseudomonas knackmussi* B13 [6–9]. The enzymes *AmnBA*, *AmnC*, *AmnE*, *AmnD*, *AmnF*, *AmnG*, *AmnH* are involved in the degradation of 2-AP into pyruvate and acetyl-CoA [1, 3, 8, 10–12]. 2-aminophenol-1,6-dioxygenase (AmnAB) converts 2-AP through an extradiol meta cleavage into 2-aminomuconic 6-semialdehyde (2-AMS). 2-AMS is further oxidized by the AmnC dehydrogenase into 2-aminomuconic acid (2-AM), which is converted by AmnD into 4-oxalocrotonic acid with a concomitant release of ammonium. 4-oxalocrotonic acid is further degraded by the enzymes AmnE, AmnF, AmnG and AmnH into pyruvate and acetyl-CoA.

*B. xenovorans* LB400 is a model bacterium for the degradation of polychlorobiphenyls (PCBs) and other aromatic compounds [13–15]. Its genome comprises a major chromosome (C1), a minor chromosome (C2) and one megaplasmid (MP) [15]. A wide range of aromatic compounds including PCBs, hydroxyphenylacetates and hydroxybenzoates are metabolized by a number of peripheral catabolic pathways into eleven central catabolic pathways [14–17]. Strain LB400 possesses the *amn* genes that encode the enzymes from the 2-AP central catabolic pathway (Fig. 1), but the...
Figure 1. Model of the aerobic metabolism of 2-aminophenol in bacteria. The substrate and product(s) of each enzyme are indicated. The non-enzymatic production of picolinic acid during 2-AP catabolism in B. xenovorans LB400 is depicted.

2-aminophenol

2-aminophenol 1,6-dioxygenase (AmnAB)

2-aminomuconate-6-semialdehyde

2-aminomuconate-6-semialdehyde dehydrogenase (AmnC)

2-aminomuconate

2-aminomuconate deaminase (AmnD)

4-oxalocrotonate

4-oxalocrotonate decarboxylase (AmnE)

2-keto-4-pentenoate

2-keto-4-pentenoate hydratase (AmnF)

4-hidroxy-2-ketovalerate

4-hidroxy-2-ketovalerate aldolase (AmnG)

piruvate + acetaldehyde

acetaldehyde dehydrogenase (AmnH)

acetyl-CoA
functionality of this pathway has not been studied. The aim of this study was to determine the functionality of the 2-AP catabolic pathway from *B. xenovorans* LB400.

**Materials and Methods**

**Chemicals**

2-Aminophenol (99% purity), nitrobenzene (>98% purity) and picolinic acid (99% purity), were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

**Bacterial Strain and Culture Conditions**

*B. xenovorans* LB400 was cultivated in Luria Bertani (LB) medium and in nitrogen-free BLKN medium [18] using glucose (10 mM) as sole carbon source at 30°C. For selective growth of strain LB400, OF basal medium agar (Oxoid) with glucose (10 mM), 2-AP (10 mM) and NH4Cl (10 mM) for 24 h at 30°C and washed twice with sodium phosphate buffer (0.02 M, pH 7.0), (1 mM) and crystal of biphenyl (1 mg) were used.

To study growth using 2-AP as carbon and nitrogen source in liquid media, LB400 cells previously cultured in BLKN medium using biphenyl (10 mM) as sole carbon source and NH4Cl (10 mM) as sole nitrogen source, harvested by centrifugation and washed twice with sodium phosphate buffer (0.02 M, pH 7.0), were incubated in BLKN minimal medium with benzoate (10 mM) and NH4Cl (10 mM) for 24 h at 30°C on a rotary shaker (150 rpm). The cells were harvested by centrifugation, washed twice with sodium phosphate buffer and suspended in BLKN medium supplemented with glucose (10 mM), 2-AP (1 mM) or NH4Cl (1 mM) in darkness. Growth was determined by measuring turbidity at 525 nm and by counting colony-forming units (CFU). The CFU were determined using a microdot method in LB plates and were calculated as the mean ± SD of at least three independent experiments. To study the growth of strain LB400 using 2-AP as nitrogen and carbon source on solid media, BLKN agar with and without glucose (10 mM) was used. 2-AP crystals (1 mg) and/or an aliquot (5 μL) of nitrobenzene (1 M) were added. The plates were inoculated with LB400 cells and incubated at 30°C for 48 h.

Minimum inhibitory concentration (MIC) of PA for strain LB400 was determined by Mueller-Hinton broth (Difco, Detroit, USA) microdilution at 5 × 10^{-5} CFU/mL according to Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Mueller-Hinton medium was supplemented with PA in the concentration range from 0.032 mM (4 μg/mL) to 4.159 mM (512 μg/mL): 0.032, 0.065, 0.130, 0.260, 0.520, 1.040, 2.079 and 4.159 mM. MIC for PA was the lowest concentration at which no LB400 colonies able to utilize biphenyl as sole carbon source were selected. The *amnB*^{-} mutant was verified by PCR amplification with primer sets M13R and AR1, M13R and CR, M13R and DR, JF and CR.

**RNA Isolation**

Total RNA was isolated from LB400 cells using an RNeasy 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. Amplification of 16S rRNA gene and *amnB* gene using specific primer sets (Table 1) were used as controls to exclude DNA contamination. The RNA concentration was quantified using a Qubit fluorometer (Invitrogen) and a Nanodrop spectrophotometer (Thermo Scientific). RNA integrity was tested by agarose (1%) gel electrophoresis.

**RT-PCR and Real-Time RT-PCR**

Reverse transcription-PCR (RT-PCR) was carried out with sequence-specific primers design in this study (Table 1) by using SuperScript One-step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, USA). The co-expression assays were performed using the primers indicated in Table 1. The cycles of amplification (35 cycles) was carried out as follows: 95°C for 1 min, 60°C for 0.5 min, 72°C for 2 min, after an initial denaturation at 95°C for 5 min. Negative and positive controls were included in each RT-PCR assay. At least two independent RT-PCR reactions for each condition were done to assess reproducibility.

For RT-qPCR, total RNA (100 ng) was transcribed with Verso cDNA kit (Thermo Scientific, Lafayette, USA). The RT-qPCR was performed according to the MIQE guidelines [21]. Real-time PCR was performed on Mx3000P qPCR system (Stratagene), using the Kapa Sybr fast qPCR master mix (2×) universal kit (Kapabiosystems, Boston, USA) and 0.3 μM of each primer. Samples were initially denatured at 95°C for 5 min. A 40-cycle amplification and quantification protocol (95°C for 15 s, 62°C for 15 s and 72°C for 15 s) with a single fluorescence measurement per cycle followed by a melting-curve program (95°C for 15 s, 25°C for 0.1 s, 70°C for 15 s and 95°C for 0.1 s) were used according to manufacturer’s recommendations. The PCR melting curves confirm the amplification of a single product for each primer pair. The BF2 and BR1 primers were used for *amnB* gene amplification, yielding a product of 208 bp. The *ftsZ* (BacA0491) gene was amplified as a reference gene, yielding an amplicon of 113 bp. A standard curve in triplicate was made with 5 serial dilutions (10 fold) for each amplicon in a linear range (9.2 ng – 0.09 pg) of LB400 genomic DNA. qPCR efficiencies were calculated from the slopes of the log-linear portion of the calibration curves, using the equation E = 10^{(-1/slope)}. The qPCR efficiency rate was 1.95 for both *amnB* and *ftsZ* genes. The *ftsZ* gene was stably expressed according to the algorithms of BestKeeper [22]. Relative *amnB* gene expression ratios were determined by the Pfaffl method [23], normalizing the gene expression to LB400 cells grown in BLKN medium with glucose as sole carbon source and NH4Cl as sole nitrogen source.

**Degradation Assay**

Resting cells (turbidity 525 nm = 0.6) were incubated in sodium phosphate buffer (30 mM, pH 7.2) with 2-AP (1 mM). Aliquots of cell suspensions were taken at different incubation times and centrifuged (19,283 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/Lichrospher 5-mm column (Supleco, Bellefonte, USA). The aqueous mobile phase

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contained 20% methanol, 10% acetonitrile and 70% sodium acetate (0.1 M) solution. The flow rate was 1 mL min\(^{-1}\). 2-AP was quantified using calibration curves with authentic standards. Resting cells experiments were performed in triplicate.

**Metabolism of 2-AP**

LB400 cells grown in BLKN medium were incubated in the presence of 2-AP (1 mM) in darkness at 30°C. Aliquots (2 mL) were collected by centrifugation (19,283 g for 2 min) at different times and cell-free supernatants were analyzed by reverse phase chromatography with a Jasco liquid chromatograph equipped with a diode array detector and a Whatman C-18 column (25 cm by 4.6 mm ID). The aqueous mobile phase contained 20% methanol, 20% acetonitrile and 0.1% phosphoric acid and the flow rate was 1.0 mL min\(^{-1}\). The compounds were monitored at 278 nm. Under these conditions, 2-AP and picolinic acid have retention times of 4.56 and 3.73 min, respectively. 2-AP and picolinic acid were quantified using commercial authentic standards.

**Bioinformatics Analysis**

The **amn** genes and the neighborhood in the genome of strain LB400 were analyzed using the Vector NTI suite 9.0 software. For the sequence alignment the database of the National Center for Biotechnology Information (NCBI/BLAST Home) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. Clustal W software was used for analysis of the sequence of **amnB** gene with selected sequences retrieved from GenBank. The software package MEGA 5.0 [24] was used for phylogenetic analyses. A phylogenetic tree was built by using the Neighbor-Joining method with a bootstrap analysis of 5000.

**Table 1. Primers used in this study.**

| Gene   | Primer   | Reference          |
|--------|----------|--------------------|
| amnR   | RF       | ACCTACCGCTATACGACTG This study (*) |
|        | RR       | TCAATACACTGTAATCCTC |
| amnJ   | JF       | CTCGGATGCTCATGCTTCCGC |
| amnA   | AF1      | GTGGTGTCGGCTAATGGGGA |
|        | AR1      | CAGCAATTGCTTTGCGGCT |
| amnB   | BF1      | CGCATCCTGCTCATGCTTCCCA |
|        | BF2      | CTACACGGCTACTTTGCGGCT |
|        | BR1      | GTTGCCAGTGCGGATGAGC |
|        | BR2      | GACCTCGGTCTGGTTGTCGA |
| amnC   | CR       | GACAGGCTCTATACGACCTC |
| amnD   | DF       | TTTTTGTCTCAGGCACAG |
|        | DR       | ATCCGGTACGCGACTG |
| amnE   | EF       | AGTCCGGCTCAGCGGCCG |
|        | ER       | GATGACATCGGACGACG |
| amnG   | GR       | CCTGAGGCAACAGATAGTTGATG |
|        | 16S rRNA | AGAAGTCGATCGGCTG |
|        | 1492r    | TACGGYATCCCTTGTGACT |
| ftsZ   | FZF1     | CGATTACGGTTCGCTGTA |
|        | FZR1     | ATCCTCGGTTCGTTTCA |
|        | M13R     | AACAGCTATGACCATG |

**Results**

**Bioinformatic Analysis of the **amn** Genes in B. xenovorans Strain LB400**

The **amn** genes from B. xenovorans LB400 are clustered at C1 (Fig. 2). The **amn** gene cluster includes the BxeA1143, BxeA1144, BxeA1145, BxeA1146, BxeA1147, BxeA1148, BxeA1149, BxeA1150, BxeA1151 and BxeA1152 genes (hereafter **amnR, amnJ, amnB, amnA, amnC, amnD, amnF, amnE, amnH and amnG** genes, respectively) and has an extension of 8,529 bp. The predicted **amn** genes of strain LB400 are listed in Table 2. The **amnRJBACD-FEHG** gene cluster encodes the enzymes of the catabolic pathway for the conversion of 2-aminophenol into pyruvate and acetyl-CoA (Fig. 1). The **amnBA** genes encode the 2-aminophenol-1,6-dioxygenase that open the 2-aminophenol ring via meta-cleavage, producing 2-AMS. The predicted **amnC** gene encodes 2-amino-muconic-6-semialdehyde dehydrogenase that oxidizes 2-AMS into 2-AM. The **amnD** gene encodes the enzyme 2-amino-muconic deaminase that converts 2-AM into 4-oxalocrotonic acid and ammonium, which could be used as nitrogen source for bacterial growth. The predicted **amnE** encodes the 4-oxalocrotonate decarboxylase, which converts 4-oxaloacetate into 2-keto-4-pentenoate. The predicted **amnF** gene encodes 2-keto-4-pentenoate hydratase that oxidizes 2-keto-4-pentenoate into 4-hydroxy-2-ketopentanoate. The predicted **amnG** gene encodes 4-hydroxy-2-ketovalerate aldolase that degrades 4-hydroxy-2-ketopentanoate into pyruvate and acetaldehyde. The **amnH** gene encodes the enzyme acetaldehyde dehydrogenase for the final conversion of acetaldehyde into acetyl-CoA. The **amnR** and **amnJ** genes are also present in the **amn** gene cluster from strain LB400. The **amnR** gene encodes a MarR-type transcriptional regulator, which probably acts as transcriptional repressor of the **amn** gene cluster. The **amnJ** gene is...
present in diverse bacterial amn gene clusters, suggesting that its product is required for the 2-AP biochemical pathway. Gaillard et al. [9] proposed that AmnJ is a ferredoxin-like protein. We have observed that the AmnJ protein has a conserved domain from the YjgF superfamily proteins that have enamine/imine deaminase activity [25–26].

The genetic neighborhood of the amn gene cluster in strain LB400 was analyzed. The genes located upstream (between BxeA1142 and BxeA1131 (clcR) genes) and downstream (between BxeA1153 and BxeA1157) of the amn gene cluster were studied (Fig. 2A). Diverse transcriptional regulator genes are located upstream of the amn gene cluster. The BxeA1141, BxeA1138, BxeA1137, BxeA1136, BxeA1135 and BxeA1134 genes encode a TetR-type transcriptional regulator closely related to a TetR-type regulator from Rhizobium leguminosarum bv. viciae USDA 2370 (accession number EJ801162; 56% identity), a TetR-type transcriptional regulator closely related to a TetR-type regulator from Acidovorax sp. JS42 (93% identity), a MarR-type transcriptional regulator closely related to a MarR-type regulator protein from Acidovorax sp. JS42 (77% identity), a LysR-type transcriptional regulator closely related to a LysR-type regulator protein from Bordetella petrii DSM 12004 (CAP41606.1; 81% identity), a XRE-type transcriptional regulator closely related to a XRE regulator protein from Alcaligenes sp. HPC1271 (EKU29625.1; 56% identity), and a LysR-type transcriptional regulator closely related to a LysR-type regulator protein from Pseudomonas pseudoalcaligenes JS45 (PPU93363), respectively (Fig. 2). Three genes located downstream of the amn gene cluster encode membrane transporter proteins. The BxeA1153, BxeA1156 and BxeA1157 genes encode three components (an outer membrane protein, an inner membrane protein and a transmembrane protein, respectively) of a major facilitator superfamily (MFS) multidrug efflux system. This multidrug efflux system possesses high identity with a multidrug efflux transporter pump from Raistonia solaniakensis GMI1000 [27], including the channel-forming component (CAD17593; 54% identity), the inner membrane protein (CAD17592; 50% identity) and the transmembrane protein (CAD17591; 57% identity), respectively. The BxeA1154 gene encodes a TraG conjugal transfer coupling protein that has 33% identity with the TraG protein (accession number AFT71063.1) from Alcanivorax dieselolei B5 [28].

The analysis of the genetic organization of the amn genes in bacteria indicate a conserved amnJBACD gene cluster encoding enzymes for the conversion of 2-AP until 4-oxalocrotonate, and amnFEHG genes encoding enzymes involved in the transformation of 4-oxalocrotonate into acetyl-CoA. Regulation of the amn gene

Figure 2. Organization of the predicted genes encoding the 2-aminophenol central pathway in B. xenovorans LB400 and comparison of bacterial amn gene clusters. A. The amn genes encoding the 2-aminophenol central pathway located at the major chromosome (C1). Genes of the amn gene cluster neighborhood are represented with white arrows. The genes located upstream of the amn gene cluster encode the following proteins: clcR, LysR-type transcriptional regulator of the 3-chlorocatechol pathway; BxeA1132, pseudogene; BxeA1134, LysR-type transcriptional regulator; BxeA1135, XRE-type transcriptional regulator; BxeA1136, LysR-type transcriptional regulator; BxeA1137, MarR-type transcriptional regulator; BxeA1138, TetR-type transcriptional regulator; BxeA1139, hypothetical protein; BxeA1140, DoxX family protein; BxeA1141, TetR-type transcriptional regulator; BxeA1142, DoxX family protein. The genes located downstream of amn gene cluster encode the following proteins: BxeA1153, putative esterase; BxeA1154, TraG conjugal transfer coupling protein; BxeA1155, outer membrane protein from MFS multidrug efflux system; BxeA1156, inner membrane protein from MFS multidrug efflux system; BxeA1157, transmembrane protein from MFS multidrug efflux system. The sizes of the genes and the intergenic regions are on scale. B. Comparison of bacterial amn gene clusters. The amn sequences (accession number) are: B. xenovorans LB400 (NC007951), P. knackmussii B13 (AJ617740), Bordetella avium 197N (NC010645), P. putida HS12 plasmid pNB1 (AF319593) and plasmid pNB2 (AF319592), P. putida W619 (CP000949), Pseudomonas sp. AP3 (AB020521), P. pseudoalcaligenes JS45 (PPU93363), Comamonas testosteroni CNB-1 plasmid pCNB1 (EP079106).

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Table 2. Identification of genes involved in the 2-aminophenol degradation pathway in *B. xenovorans* LB400.

| ORF     | Gene | Orientation | Size (aa) | Function                                                                 | Homology* (source)                                                                 | Accession number | E value b | Amino acid identity % aa range |
|---------|------|-------------|-----------|--------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------|-----------|--------------------------|
| BxeA1143 | amnR | R           | 205       | MarR-family transcriptional regulator                                    | MarR-family transcriptional regulator (*Bordetella avium* 197N)                    | ABE31805.1       | 1.0 E-110 | 79 154                   |
| BxeA1144 | amnJ | F           | 137       | Putative imino/enamina deaminase                                         | Putative ferredoxin (*Pseudomonas putida* pNB1)                                   | ABE31804.1       | 1.0 E-27  | 49 136                   |
| BxeA1145 | amnB | F           | 304       | 2-Aminophenol 1,6-dioxygenase, β subunit                                  | 2-Aminophenol 1,6-dioxygenase β subunit (*Bordetella avium* 197N)                 | ABE31803.1       | 1.0 E-162 | 89 300                   |
| BxeA1146 | amnA | F           | 270       | 2-Aminophenol 1,6-dioxygenase, α subunit                                  | 2-Aminophenol 1,6-dioxygenase α subunit (*Bordetella avium* 197N)                 | ABE31802.1       | 1.0 E-119 | 74 202                   |
| BxeA1147 | amnC | F           | 492       | 2-Aminomuconate-6-semialdehyde dehydrogenase                             | 2-Aminomuconate-6-semialdehyde dehydrogenase (*Bordetella avium* 197N)           | ABE31801.1       | 0.0       | 88 434                   |
| BxeA1148 | amnD | F           | 145       | 2-Aminomuconate deaminase                                                 | 2-Aminomuconate deaminase (*Bordetella avium* 197N)                               | ABE31800.1       | 1.0 E-62  | 83 121                   |
| BxeA1149 | amnF | F           | 269       | 2-Keto-4-pentenoate hydratase                                             | 2-Keto 4-pentenoate hydratase (*Alcaligenes sp. O-1*)                             | ABE31799.1       | 7.0 E-95  | 63 166                   |
| BxeA1150 | amnE | F           | 254       | 4-Oxaloctonate decarboxyase                                               | 4-Oxaloctonate decarboxyase (*Burkholderia multivorans CGB2M*)                    | ABE31798.1       | 6.0 E-94  | 71 174                   |
| BxeA1151 | amnH | F           | 313       | Acetaldehyde dehydrogenase                                                | Acetaldehyde dehydrogenase (acylating) (*Comamonas testosteroni CNB1 pCNB1*)     | ABE31797.1       | 1.0 E-143 | 83 262                   |
| BxeA1152 | amnG | F           | 345       | 4-Hydroxy 2-ketovalerate aldolase                                          | 4-Hydroxy-2-ketovalerate aldolase (*Comamonas testosteroni CNB1 pCNB1*)           | ABE31796.1       | 1.0 E-164 | 87 290                   |

*The amn genes from *B. xenovorans* LB400 and *P. knackmussii* B13 are almost identical; therefore the identity between them are not listed.

bE values are based on BLASTP results of the non-redundant NCBI database.

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cluster is generally mediated by an amnR gene-encoded Mar-type family transcriptional regulator located upstream of the amn gene cluster. *B. xenovorans* LB400, *P. knackmussii* B13 [9] and *Bordetella avium* 197N [29] shared the amnRJBACD gene organization (Fig. 2). *P. putida* strains HS12 and W619 shared the amnRJBACD gene cluster, whereas the amnD gene is located after the amnFE genes. *P. pseudoalcaligenes* strain JS45 and *Pseudomonas* sp. strain AP-3 showed a similar amnRJBACD gene organization, but in these strains the amnR gene and amnR genes have not been reported (Fig. 2). In *Pseudomonas* sp. strain AP-3 the amnD gene is located between amnF and amnE genes. *Comamonas testosteroni* strain CNB-1 possesses a similar gene cluster for the 2-amino-5-chlorophenol catabolism [30]. The cahBACD gene cluster that encodes the enzymes for the conversion of 2-amino-5-chlorophenol into 4-oxalocrotonate has the same gene organization (Fig. 2). The genetic organization of amnFEGH genes, which encode the enzymes for the conversion of 4-oxalocrotonate into acetyl-CoA and is conserved in other bacteria, is located in *B. xenovorans* strain LB400 downstream of the amnRJBACD gene cluster. However, in *Pseudomonas* strains HS12, W619 and AP-3, the amnD gene is located within the amnFEGH cluster (Fig. 2). The amnFEGH region has not been reported in the amn gene cluster of *P. pseudoalcaligenes* strain JS45.

The phylogenetic sequence analysis of the 2-amino-phenol 1,6-dioxygenase AmnB β-subunits and related class III ring-cleavage dioxygenases is illustrated in Figure 3. The 2-amino-phenol 1,6-dioxygenase β-subunits were clustered in two branches. One branch includes the AmnB proteins of *B. xenovorans* LB400, *P. knackmussii* B13, *B. avium* 197N and *Achromobacter xylosoxidans* A8. The second branch comprises 2-amino-phenol 1,6-dioxygenase β-subunits from *Pseudomonas* sp. AP-3, *P. putida* W619, *P. putida* HS12 and *P. pseudoalcaligenes* JS45. This sequence analyses suggest that AmnB protein of *B. xenovorans* LB400 evolved separately from AmnB protein of *Pseudomonas* sp AP-3 and *P. pseudoalcaligenes* strain JS45. This is in accordance with the genetic organization of amn gene clusters (Fig. 2). The gene organization of amnRJBACD cluster of *B. xenovorans* strain LB400, *P. knackmussii* B13, *B. avium* 197N is identical, and their AmnB enzymes were classified in the same branch in the phylogenetic tree. *P. pseudoalcaligenes* JS45 and *Pseudomonas* sp. AP-3 showed a different amn gene cluster than *B. xenovorans* strain LB400 and also their AmnB proteins were classified in a different phylogenetic branch. The AmnB proteins are closely related to the 2-amino-3-chlorophenol-1,6-dioxygenase CusCβ proteins that oxidizes 4-chloro-2-amino-phenol, but less related to dioxygenases subunits involved in the oxidation of 3,4-dihydroxyphenylacetae and 2’-amino-4-phenyl-2,3-diol. All these dioxygenases shared a common ancestor with catechol 2,3-dioxygenase NahH and XylE proteins. Phenoxazinone synthases, which use the same substrate as AmnB proteins, are closely related to catechol 1,2-dioxygenases but not related to 2-amino-phenol 1,6-dioxygenase AmnB β-subunits.

**Transcription of the amn Genes**

The transcription of the amn genes in strain LB400 was studied using specific primer sets (Fig. 4A). To study the co-expression of the amn genes, diverse primers sets were designed to amplify adjacent genes. RT-PCR of mRNA from LB400 cells grown until exponential phase in BLKN medium with glucose and 2-AP as sole carbon and nitrogen sources were performed. The expression of the amnR gene in strain LB400 was observed (Fig. 4B). Two divergent σ9-type promoters were identified in the amn gene cluster, which are located between the amnR gene and the amnF gene. RT-PCR analyses showed the co-expression for amnF, amnB, amnACD, amnDFE and amnEHG genes (Fig. 4B), indicating that the amn gene cluster is an operon in strain LB400. The co-expression of the amn genes strongly suggests that the promoter located upstream of the amnF gene permits the expression of an amnFamnBACDEFG single transcript. A putative MarR-type regulatory binding sequence (TAGTGACGTAACGTTATC) was identified upstream of the amnFamnBACDEFG gene cluster, which showed a consensus MarR-type regulator binding sequence (5′-TAG(T/G)/ACGTAACGTTAC-3′) located upstream of the amnF gene from diverse 2-AP-degrading bacteria: *P. putida* W619, *C. testosteroni* CNB1, *P. pseudoalcaligenes* JS45, *P. knackmussii* B13 and *Bordetella avium* 197N. The amnB gene encodes the 2-amino-phenol 1,6-dioxygenase beta subunit, which is the key enzyme of 2-AP catabolic pathway. The expression of amnB gene in LB400 cells grown in glucose in presence or absence of 2-AP and nitrobenzene was studied. Real time RT-PCR analysis showed that the amnB gene has a basal expression during growth in glucose. The amnB gene expression was highly induced by 2-AP, whereas lower induction was observed in cells during exposure to 2-AP and glucose (Fig. 4C). The lowest induction of amnB gene expression was observed in cells during exposure to nitrobenzene. Overall these results indicate a regulated expression of the amn gene cluster in strain LB400.

**2-amino-phenol Degradation**

To further characterize the 2-AP catabolic pathway, 2-amino-phenol degradation by strain LB400 was studied. Resting LB400 cells were incubated with 2-AP. LB400 cells degrade 15% of 2-AP after 6 h of incubation, whereas complete degradation was observed after 30 h (Fig. 5). In contrast, 2-AP was not degraded by boiled LB400 cells. The amnR− mutant of strain LB400 is not able to degrade 2-AP, indicating that 2-amino-phenol-1,6-dioxygenase is the sole enzyme involved in 2-AP degradation in strain LB400.

**Growth of Strain LB400 on 2-amino-phenol**

The expression of the amn genes and the degradation of 2-AP, suggest a functional 2-AP catabolic pathway in *B. xenovorans* strain LB400. In a further analysis of this metabolic pathway, the growth of strain LB400 in minimal medium with 2-AP as nitrogen or carbon source was studied. In a first approach, the growth of strain LB400 on BLKN agar plates in presence of 2-amino-phenol crystals in absence or presence of glucose was studied. Small colonies of LB400 cells grown around the 2-AP crystals used as sole nitrogen source or as sole carbon and nitrogen source on BLKN plates were observed (data not shown). An increased number of small colonies around the 2-AP crystals on plates with 2-AP and supplemented with nitrobenzene was observed, suggesting that nitrobenzene stimulate growth probably by the induction of the 2-AP catabolic pathway genes. These small colonies were phenotypically confirmed as *B. xenovorans* LB400. The bacteria of the colonies were able to grow in minimal medium supplemented with biphenyl as sole carbon and energy source and show typical colonies on OFBPL medium agar (data not shown). In contrast, no colonies were observed on plates with nitrobenzene as sole carbon and nitrogen source (data not shown).
In a second approach, the growth of strain LB400 in liquid BLKN medium with 2-AP (1 mM) as sole nitrogen source or as sole nitrogen and carbon source was analyzed. Growth of LB400 cells in BLKN medium with 2-AP as sole nitrogen source and...
glucose as carbon source was observed (Fig. 6A). However, the cells reached lower biomass than cells incubated with NH4Cl as sole nitrogen source and glucose as sole carbon source. These results suggest that an inhibitory metabolite was produced during LB400 growth on 2-AP and glucose. LB400 cells showed a minimal growth using 2-AP as sole carbon and nitrogen source, which was similar to growth in absence of other carbon and nitrogen source, suggesting that in absence of other carbon and nitrogen source, 2-AP could be used as carbon source, but not as nitrogen source. No LB400 growth was observed in presence of NH4Cl and absence of a carbon source (Fig. 6A). To link the LB400 growth on 2-AP with the \textit{amn}-encoded catabolic pathway, the growth on 2-AP of an \textit{amnBA} \textit{B. xenovorans} LB400 mutant derivative of \textit{B. xenovorans} LB400 generated by homologous recombination was studied. The \textit{amnBA} mutant was unable to grow in BLKN medium with 2-AP as sole nitrogen source and glucose as carbon source, which correlates with no degradation of 2-AP (data not shown).

To study if during the degradation of 2-AP by strain LB400 a toxic metabolite was produced, the supernatants of cultures mentioned above were analyzed by HPLC. 2-AP was degraded during the first 15 h, independently if 2-AP was used as nitrogen or as carbon source (Fig. 6B). Interestingly, 2-AP degradation correlates with the production of a metabolite. This metabolite was identified as picolinic acid (PA) by comparison of its absorption spectrum and HPLC retention time with an authentic standard. Picolinic acid could be produced by a spontaneous non-enzymatic reaction than convert 2-AMS into PA. 2-AMS is produced from 2-AP by the 2-aminophenol-1,6-dioxygenase. During 2-AP degradation only partial conversion into PA was observed. The maximal PA concentration reached during growth using 2-AP (1 mM) as carbon source or as carbon and nitrogen source was approximately 0.5 mM, which correlates with a low cell growth. When 2-AP was used as sole nitrogen source a higher biomass was reached whereas a lower PA concentration (0.25 mM) was observed.

Figure 7 illustrate the effects of 2-AP and PA on \textit{B. xenovorans} strain LB400 growth. LB400 growth on glucose and NH4Cl was not affected by the addition of 2-AP at late exponential phase (Fig. 7A). In contrast, when 2-AP was present since lag phase, a low cell density was reached at stationary phase. These results suggest that 2-AP has to be metabolized to exert an inhibitory effect on LB400 growth. On the other side, the effect of the addition of PA on LB400 growth was studied (Fig. 7B). The addition of PA at the lag phase reduced significantly the biomass reached at exponential phase of LB400 culture growing with glucose and NH4Cl. However, the addition of PA at late exponential phase did not affect the cell growth. The addition of 2-AP or PA at mid-exponential phase of LB400 cultures growing with glucose and NH4Cl inhibited also the cell growth (Fig. 7C). Finally, the minimal inhibitory concentration (MIC) of PA for \textit{B. xenovorans} strain LB400 was measured. The MIC of PA that prevented LB400 growth was 2 mM.
Discussion

This study revealed that the 2-aminophenol catabolic central pathway is functional in the model aromatic-degrading bacterium *B. xenovorans* LB400. Strain LB400 is able to use 2-AP as sole nitrogen source for growth, indicating an active 2-AP catabolic central pathway. Only few bacterial strains capable to grow on 2-AP have yet been reported [5,6]. *Pseudomonas* sp. strain AP-3 is able to grow in liquid medium using 2-AP as sole carbon and nitrogen source [5]. *P. pseudoalcaligenes* strain JS45 grow in liquid medium using 2-AP as sole carbon and nitrogen source but only when the cells were previously induced with NB [6]. In contrast, the nitrobenzene-degrader *Pseudomonas putida* strain HS12 did not grow on 2-AP [7]. Interestingly, *P. knackmussi* strain B13 that share a highly identical *amn* gene cluster with *B. xenovorans* LB400 is not able to grow on 2-AP [9]. In our study, strain LB400 reached lower cell numbers in liquid medium using as sole nitrogen source for growth 2-AP (2.5 ± 10⁸ CFU mL⁻¹) than ammonium chloride (4.5 ± 10⁸ CFU mL⁻¹). On the other side, only minimal growth of strain LB400 was measured in liquid medium with 2-AP as sole nitrogen and carbon source. In addition, small colonies of strain LB400 were observed on agar plates using 2-AP crystals as sole nitrogen and carbon source. All these results indicate a reduced growth of strain LB400 on 2-AP, suggesting an inhibition of cell growth during the metabolism of 2-AP. The growth inhibition suggests the production of a toxic metabolite. In this study, we were able to demonstrate that during the degradation of 2-AP by strain LB400 this substrate is partially converted into the antibiotic picolinic acid. When 2-AP (1 mM) was used as sole carbon and nitrogen source for strain LB400 growth, high levels of PA (0.5 mM) were produced. In this report we showed that PA inhibited the growth of *B. xenovorans* strain LB400. The MIC of PA for strain LB400 is 2 mM. PA is a well known antibiotic for Gram-negative bacteria such as *P. aeruginosa* and *Staphylococcus aureus*, *Mycobacterium avium* complex and fungi such as *Candida albicans* [31–33]. PA is a chelating agent for metals ions such as Fe²⁺ and Zn²⁺.

Interestingly, it has been reported that PA induces antitumoral activity of macrophages and chromium picolinate has been used as a dietary supplement for obese persons to promote weight loss [31–36]. Nishino and Spain [6] reported that crude extracts of *P. pseudoalcaligenes* strain JS45 transformed 2-AP through 2-AMS into PA. The addition of NAD to the extracts of strain JS45 prevented the formation of PA by favoring the formation of 2-aminomuconate by the 2-aminomuconic-6-semialdehyde dehydrogenase AmnC. For the production of PA, a recombinant *E. coli* strain harboring only the genes for 2-aminophenol 1,6-dioxygenase from strain JS45 was used for the biotransformation of 2-AP into PA [37]. *P. knackmussi* strain B13 that has a highly identical 2-AP catabolic pathway as strain LB400 did not grow on 2-AP [9]. To explain the failure of strain B13 growth on 2-AP, Gaillard *et al.* proposed the production of a toxic intermediate from 2-AP or alternatively a metabolic misrouting. Our study demonstrates the conversion of 2-AP into PA by the *amn*-encoded catabolic pathway in *B. xenovorans* strain LB400. These results suggest that strain B13 also produces the toxic PA during 2-AP degradation, impeding its growth on 2-AP. The biotransformation of aromatic compounds into toxic metabolites and antibiotics has been reported [38–41]. Câmara *et al.* [40] reported that PCBs were converted by enzymes.
of the biphenyl catabolic pathway into highly toxic biphenyl dihydrodiols and dihydroxylated biphenyls. Blasco et al. [38,39] demonstrated that chlorobenzoates were metabolized by bacteria through 4-chlorocatechol into the antibiotic protoanemonin.

Genome analysis indicates that the amn genes for 2-aminophenol degradation are clustered in the clc genomic island at C1 from *B. xenovorans* LB400. LB400 clc genomic island shows 99% sequence identity with the clc element of *P. knackmussii* strain B13.
with glucose (10 mM) as sole carbon source and NH₄Cl (1 mM) as sole nitrogen source. Two cultures were supplemented with 2-AP (1 mM) at time 0 or at late exponential phase (18 h). As controls, LB400 cells were cultured in M9 minimal medium without a carbon source or cultured with glucose. B, LB400 cells were cultured in BLKN minimal medium with glucose (10 mM) and NH₄Cl (1 mM) as sole carbon and nitrogen source, respectively. Two cultures were supplemented with picolinic acid (1 mM) at time 0 or at late exponential phase (18 h). C, LB400 cells cultured in BLKN minimal medium with glucose (10 mM) and NH₄Cl (1 mM), were supplemented after 13 h with 2-AP (1 mM) or PA (1 mM).

The knockout strain B13 have the same energy source [6,42]. and contains genes of the 2-aminophenol and chlorocatechol catabolic pathways and genes involved in the transport of aromatic compounds. The amn gene clusters from B. xenovorans LB400 and P. knackmussi strain B13 have the same amn gene organization and the ann genes of both strains have almost identical gene sequence [9], which strongly suggest horizontal gene transfer of the amn gene cluster between bacteria. Genome analysis of strain LB400 indicates the absence of nbz (hab) genes involved in the conversion of nitrobenzene into 2-aminophenol. In accordance with the genome analysis, strain LB400 was unable to grow using nitrobenzene as sole nitrogen or carbon source. In contrast, both 2-AP-degrading Pseudomonas strains JS45 and HS12 possess the nbzA (habA) and nbzB (habB) genes for nitrobenzene degradation and are able to grow on nitrobenzene as carbon and nitrogen energy source [6,42].

The 2-aminophenol-1,6-dioxygenase AmnB subunit of B. xenovorans LB400 is closely related to 2-amino-5-chlorophenol-1,6-dioxygenase CubCh subunit of C. testosteroni strain CNB-1 (70% identity). In addition, the organization of the cub gene cluster of C. testosteroni CNB-1 and the ann gene cluster of P. putida HS12 is conserved, suggesting a common origin. The phenoazinone synthase (GriF, PhsA or NspF) of several members of the Streptomyces genus that use also 2-AP as substrate are not related to 2-aminophenol-1,6-dioxygenase [44]. This is not surprising since phenoazinone oxidase is not an extradiol dioxygenase.

In this study, we showed that B. xenovorans strain LB400 grew using 2-AP as sole nitrogen source and that LB400 cells degraded completely 2-AP. In contrast, an annBA− mutant was unable to grow on 2-AP as nitrogen source and to degrade this compound, linking the ann-encoded central pathway with 2-AP catabolism in strain LB400. In addition, RT-PCR analysis showed that annA, annB, annC, annD, annE, annF, annG, annH, annJ and annR genes were expressed in LB400 cells. These results demonstrate that the ann gene cluster is functional. The co-expression of the annJB, annBAC, annACD, annDFE and annEHG genes and the presence of a promoter upstream of the annJ gene indicate that the ann genes are clustered in an operon. The expression of the annB gene in LB400 cells grown in glucose indicates a basal expression of the ann gene. The annB gene expression is highly induced by 2-AP, whereas lower induction was observed in presence of 2-AP and glucose. These results suggest an induction of the annB gene transcription by 2-AP or its metabolic intermediates and a down regulation of its transcription by glucose. In accordance with the regulation of the ann genes observed in our study, transcriptional analysis with a DNA chip showed a higher expression of the ann genes of strain LB400 in benzoate than in succinate [45]. The predicted annR gene-encoded MarR-type transcriptional regulator

Figure 7. Effects of 2-aminophenol and picolinic acid on B. xenovorans LB400 growth. A, LB400 cells were cultured in M9 minimal medium with glucose (10 mM) as sole carbon source and NH₄Cl (1 mM) as sole nitrogen source. Two cultures were supplemented with 2-AP (1 mM) at time 0 or at late exponential phase (18 h). As controls, LB400 cells were cultured in M9 minimal medium without a carbon source or cultured with glucose. B, LB400 cells were cultured in BLKN minimal medium with glucose (10 mM) and NH₄Cl (1 mM) as sole carbon and nitrogen source, respectively. Two cultures were supplemented with picolinic acid (1 mM) at time 0 or at late exponential phase (18 h). C, LB400 cells cultured in BLKN minimal medium with glucose (10 mM) and NH₄Cl (1 mM), were supplemented after 13 h with 2-AP (1 mM) or PA (1 mM).

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of strain LB400 is probably a transcriptional repressor of the amn gene cluster. MarR-type transcriptional regulators bind as dimers to palindromic sequences within the promoter. A regulatory palindromic sequence was observed in the intergenic region of the amnR gene and the amnJBAFDEFGH gene cluster from strain LB400. MarR regulators bind to DNA in absence of the specific ligand, causing generally transcriptional repression [46]. P. knackmussi strain B13 showed by dot blot hybridization a basal amnB gene expression in glucose and a 50-fold increase amnB gene expression in 2-AP (1 mM) and antrhanilate (1 mM) [9]. P. putida HS12 down regulated the amn genes in succinate [7]. In contrast, Pseudomonas sp. strain AP3 expresses constitutively the amn genes [8], which correlates with the absence of the amnR gene in its amn gene cluster.

Figure 1 illustrates the predicted functional 2-AP catabolic pathway of the model bacterium B. xenovorans strain LB400. 2-Aminophenol-1,6-dioxigenase encoded by the amnB1 genes meta-cleaved 2-aminophenol into 2-AMS. The amnB1 gene product is not able to degrade 2-AP, indicating that 2-aminophenol-1,6-dioxigenase is the sole enzyme involved in 2-AP degradation in strain LB400. 2-AMS is further oxidized by 2-aminomuconic-6-semialdehyde dehydrogenase AmnC to 2-AM. Alternatively, 2-AMS could chemically rearrange into PA (Fig. 1). The 2-AM is deaminated by 2-aminomuconic deaminase AmnD releasing 4-oxaloacetic acid and ammonium. The ammonium released at this step could be used as nitrogen source for bacterial growth. 4-oxaloacate is converted into pyruvate, acetaldehyde and acetil-CoA, by catabolic enzymes that are also involved in the catechol metabolism.

In conclusion, this study indicates that B. xenovorans strain LB400 possess a functional 2-AP catabolic pathway, which channel the aromatic pollutant 2-AP into the Krebs cycle. Interestingly, the 2-AP degradation in strain LB400 could lead to the production of the antibiotic picolinic acid, which inhibits its growth. The versatile catabolic repertoire for aromatic compounds of B. xenovorans LB400 allows the degradation of aromatic pollutants with chloro or amino substituent’s such as PCBs and 2-AP, which could be useful for bioremediation studies.

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Author Contributions

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

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