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Modified 3-oxoadipate pathway for the biodegradation of methylaromatics

Running title: modified 3-oxoadipate pathway

Macarena Marín[1], Danilo Péerez-Pantoja[2], Raul Donoso[2,4], Victor Wray[3], Bernarndo González[2,4], Dietmar H. Pieper*[1].

[1]Department of Microbial Pathogenesis, HZI – Helmholtz Zentrum für Infektionsforschung, Braunschweig, Germany

[2] Departamento de Genética Molecular y Microbiología. Facultad de Ciencias Biológicas, NM-EMBA, CASEB, P. Universidad Católica de Chile, Santiago, Chile

[3]Division of Structural Biology, HZI - Helmholtz Zentrum für Infektionsforschung, Braunschweig, Germany

[4] (Departament, division?)Facultad de Ingenieria y Ciencias. Universidad Adolfo Ibáañez, Santiago, Chile.

*Address Correspondence to: Dietmar H. Pieper, Department of Microbial Pathogenesis, HZI – Helmholtz Zentrum für Infektionsforschung, Inhoffenstraße 7, 38124 Braunschweig, Germany. Phone: +49 (531) 61814200, Fax: +49 (531) 61814499. E-mail: dpi@helmholtz-hzi.de

Abstract

Catechols are central intermediates in the metabolism of aromatic compounds. Degradation of 4-methylcatechol via *ortho* (intradiol)-cleavage usually leads to the formation of 4-methylmuconolactone (4-ML) as a dead-end metabolite. Only a few microorganisms are known to mineralize 4-ML. The *mml* gene cluster of *P. reinekei* MT1, which encodes enzymes involved in the metabolism of 4-ML is shown here to encode 10 genes found in a 9.4 kb chromosomal region. Reverse transcription assays revealed that these genes form a single operon, where their expression is controlled by two promoters and are induced in the presence of 4-methylsalicylate, 4-ML or 3-methylmuconolactone (3-ML). Mineralization of 4-ML is initiated by the 4-methylmuconolactone methylisomerase encoded by *mmlI*. This reaction produces 3-ML and is followed by a rearrangement of the double bond, catalyzed by the methylmuconolactone isomerase encoded by *mmlJ*. Deletion of *mmlL* encoding a protein of the metallo-β -lactamase superfamily resulted in loss of the capability of strain MT1 to open the lactone ring, indicating its function as 4-methyl-3-oxoadipate enol-lactone hydrolase. Further metabolism can be assumed to occur by analogy with reactions known from the 3-oxoadipate pathway. *mmlF* and *mmlG* probably encode a 4-methyl-3-oxoadipyl-CoA transferase and the *mmlC* gene product functions as a thiolase transforming 4-methyl-3-oxoadipyl-CoA into methylsuccinyl-CoA and acetyl-CoA, as indicated by the accumulation of 4-methyl-3-oxoadipate in the respective deletion mutant. Accumulation of methylsuccinate by a *mmlK* deletion mutant indicates that the encoded acetyl-CoA hydrolase/transferase is crucial for channeling methylsuccinate into the central metabolism.

Abbreviations: Cb, carbenicillin; Gm, gentamicin; Tc, tetracycline; Sp, spectinomycin; Km, kanamycin; 4-ML, 4-methylmuconolactone; 3-ML, 3-methylmuconolactone; PFGE, pulse field gel electrophoresis; CHEF, contour-clamped homogeneous electric field

electrophoresis; ORF, open reading frame; HPLC, high-performance liquid chromatography.

Introduction

Aromatic compounds are among the most widely distributed organic substances in nature. They are present as aromatic amino acids and as constituents of fossil fuels and lignin. Microorganisms have developed the ability to use an impressive variety of such chemical compounds as carbon and energy sources (24, 60). An extensive array of substituted aromatic structures are transformed to a few central intermediates to be subject of ring cleavage (9, 26).

Catechol is one of the most important central intermediates in the aerobic metabolism of aromatic compounds, such as salicylate, benzoate, phenol, mandelate and anthranilate, among others (26). This intermediate can be channeled into the Krebs cycle by *ortho* (intradiol)-cleavage via the 3-oxoadipate pathway, which is a widely distributed route among soil bacteria (26). In this pathway, the aromatic ring is cleaved by a catechol-1,2-dioxygenase resulting in the formation of *cis,cis*-muconate, which is subsequently transformed by a muconate cycloisomerase to muconolactone. This intermediate is further transformed to 3-oxoadipate-enol-lactone by a muconolactone isomerase. Subsequently, the enol-lactone is hydrolyzed by a enol-lactone hydrolase and the resulting 3-oxoadipate in turn is channeled by 3-oxoadipate:succinyl-CoA transferase and 3-oxoadipyl-CoA thiolase to the Krebs cycle (Fig. 1). However, the 3-oxoadipate pathway is not suited for the degradation of methylaromatics. If 4-methylcatechol is subjected to *ortho*-cleavage, 4-methylmuconolactone (4-ML) forms and accumulates, as muconolactone isomerases require a proton at the C_4 carbon atom to catalyze the isomerization to enollactone (10, 33). Most bacteria described so far mineralize methylaromatics via the alternative *meta* (extradiol)-cleavage pathway (37, 55).

Until recently, only two bacteria (*Cupriavidus necator* JMP134 (46) and *Rhodococcus rhodochrous* N75 (5, 11)) had been reported to degrade 4-methylcatechol via an *ortho*-

cleavage pathway and to be able of 4-ML mineralization. *C. necator* JMP134 harbors the *mml* gene cluster (CP000090: ReutA1502-A1508), which has been proposed to comprise seven open reading frames (ORFs) encoding enzymes and putative proteins involved in the metabolism of 4-ML (22, 45). Only 4-methylmuconolactone methylisomerase (MmlI) and methymuconolactone isomerase (MmlJ), encoded by the *mmlI* and *mmlJ* genes, respectively, have a described function (49, 53). By sequence comparison with this gene cluster, *Cupriavidus necator* H16 was also found to harbor a putative *mml* gene cluster (AY305378: PHG384-390). However, whether this cluster is functional or not remains to be elucidated.

~~As shown in figure 1, the~~The degradation of 4-ML in both *C. necator* JMP134 and *R. rhodochrous* N75 is initiated by MmlI (Fig. 1), which catalyzes the isomerization of 4-ML to 3-ML (5, 49). In *C. necator* JMP134, further degradation is accomplished by MmlJ, which by analogy with the 3-oxoadipate pathway transforms 3-ML to 4-methyl-3-oxoadipate enol-lactone (52). As well, it has been proposed that in this strain the enol-lactone intermediate may be transformed to 4-methyl-3-oxoadipate by a hydrolase (26, 46). However, no typical enol-lactone hydrolase activity towards methyl-substituted muconolactones has been observed so far (52).

Similar to *C. necator* JMP134, *R. rhodochrous* N75 degrades 4-methylcatechol via 4-ML (Fig. 1) (5). ~~However~~In contrast, in *R. rhodochrous* N75 ~~this organism,~~ 3-ML is directly activated by a 3-methylmuconolactone-CoA synthetase, which catalyzes the synthesis of 3-ML-CoA from ATP, coenzyme A and 3-ML (11). Unfortunately, no gene or protein sequence data related to this transformation are available. Further degradation of 3-ML-CoA has been proposed to proceed via 4-methyl-3-oxoadipyl-CoA, although details of this reaction are not available.

Recently, C~~a~~áamara *et al*. reported that *Pseudomonas reinekei* MT1 degrades 4-methylsalicylate via *ortho*-cleavage of 4-methylcatechol (7). This strain harbors a gene

cluster encoding a salicylate 1-hydroxylase (SalA), a catechol 1,2-dioxygenase (SalD) and a muconate cycloisomerase (SalC). Both, SalD and SalC are specialized for the transformation of methyl-substituted substrates ensuring effective funneling of methylaromatics into the *ortho*-cleavage pathway. Additionally, *P. reinekei* MT1 exhibits MmlI activity (7), which indicates that methylsubstituted aromatics are degraded via 4-ML. In contrast to *C. necator* JMP134, which mineralizes methylaromatics such as 4-methylphenol mainly via a *meta*-cleavage pathway despite the functionality of the *ortho*-cleavage pathway (47), *P. reinekei* MT1 relies solely on the *ortho*-cleavage route to mineralize methycatechols and thus represents an ideal system to study this pathway in detail (7).

In this report we describe in *P. reinekei* MT1 a gene cluster encoding proteins involved in the degradation of 4-ML ~~in *P. reinekei* MT1~~ and analyze the operonic organization and expression profile of these genes. Based on genetic data and on analysis of metabolites produced and accumulated in different deletion mutants we were able to reconstruct the metabolic pathway encoded by this gene cluster.

Material and Methods

Chemicals. 4-ML, 3-ML and 5-chloro-3-methylmuconolactone were prepared as described earlier (33, 46, 48).

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in table S1. *P. reinekei* strain MT1 was grown in minimal medium as previously described (39), with 5 mM salicylate or 4-methylsalicylate as sole carbon source. *C. necator* JMP134::X, a derivative of *C. necator* JMP134 engineered to catabolize 4-methylbenzoate by chromosomal insertion of the *xylXYZL* genes encoding a

broad substrate range toluate 1,2-dioxygenase and a toluate dihydrodiol dehydrogenase (35), was grown in the same medium with 2.5 mM 4-methylbenzoate as sole carbon source. Luria-Bertani (LB) medium was used as rich medium for *Escherichia coli*, *P. reinekei* and *C. necator* strains. For selection of mutants, ABC medium (AB medium (16) supplemented with trace metals (20) and 20 mM citrate) was used. Antibiotics were used at the following concentrations: for *E. coli*, Cb (100 µg/mL); Gm (10 µg/mL); Tc (10 µg/mL); Sp (100 µg/mL), for *P. reinekei*, Gm (200 µg/mL); Tc (15 µg/mL); Sp (100 µg/mL) and for *C. necator* JMP134, Gm (20 µg/mL); Sp (100 µg/mL) and Km (100 µg/mL).

Enzymatic assays. Cell extracts of *P. reinekei* MT1 grown on 4-methylsalicylate were prepared as previously described (39). MmlI activity was measured by high-performance liquid chromatography (HPLC) following the transformation of 4-ML into 3-ML as reported previously (49). Activity of MmlJ was determined spectrophotometrically, measuring the transformation of 200 µM 5-chloro-3-methylmuconolactone in 50 mM Tris-HCl buffer pH 7.5, as previously described (53). One unit (U) was defined as µmoles of product formed per minute.

Partial purification of MmlJ and N-terminal sequence determination. MmlJ was partially purified by anion exchange chromatography using a MonoQ HR 5/5 column (Amersham Pharmacia Biotech). Cells extracts were applied directly onto the column and proteins eluted by a linear gradient of 0 – 0.5 M NaCl over 33 mL with a flow of 0.5 mL/min. MmlJ eluted at 0.37 ± 0.01 M NaCl. Aliquots of highly active fractions were subjected to SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and major protein bands of a molecular mass of ~ 10 kDa were analyzed by N-terminal sequencing (30).(29)(29)(29)(29)(29)(29)(30)(29)(30)(29)(31)(31)(31)(30)(30)(30)

Fosmid library screening, sequencing and sequence analysis. In order to localize the *mml* gene cluster, part of the *mmlJ* gene was amplified by PCR using degenerate primers

NH3MMLIF1 and NH3MMLIR1, which were designed based on the N-terminal protein sequence of the partially purified MmlJ from *P. reinekei* MT1. Primer sequences are shown in table S2. The approximately 75 bp PCR product generated was cloned into the pGEM-T Easy vector (Promega), transformed into *E. coli* Max Efficiency DH5α competent cells (Invitrogen) and sequenced. Based on the cloned sequence, a specific forward primer NH3MMLIF3 was designed and ~~was~~ used in a second PCR round with a reverse degenerated primer NH3MMLIR4 designed from a sequence alignment of methylmuconolactone and muconolactone isomerases. The generated 125 bp fragment was cloned in the pGEM-T Easy vector, transformed into *E. coli* JM109 (Stratagene) and sequenced. A previously constructed fosmid library of *P. reinekei* MT1 genomic DNA (7) was screened by PCR using primers NH3MMLIF3 and NH3MMLIR7 specific for *mmlJ* gene. Positive fosmid clones were purified with the FosmidMAX DNA purification kit (Epicentre) and subjected to direct sequencing of the upstream and downstream the *mmlJ* gene flanking regions, using the ABI PRISM BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Raw sequence data from both strands were assembled manually.

DNA and protein similarity searches were performed using BLASTX and BLASTP programs from the NCBI website (3). Translated protein sequences were aligned with MUSCLE using default values (21). Phylogenetic trees were constructed with MEGA4 (58) using the n~~N~~eighbor-j~~J~~oining algorithm (54) with p-distance correction and pairwise deletion of gaps and missing data. A total of 100 bootstrap replications were done to test for branch robustness.

Extrachromosomal DNA extraction. Detection of megaplasmids was attempted by pulse field gel electrophoresis (PFGE). *P. reinekei* MT1 was cultivated at 30ºC in 100 mL LB medium to an A_{600}=0.5. Cells were harvested by centrifugation and resuspended in SE

solution (75 mM NaCl, 25 mM EDTA, pH 8). To avoid shearing of high molecular mass DNA, cells were mixed with an equal volume of 2% (w/v) low melting point agarose (Invitrogen). The mixture was poured into plugs, which were incubated over night at 50ºC with 0.5 mg/mL proteinase K. To inhibit the protease, the plugs were incubated in TE buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8) with 1 mM Pefabloc Sc (AEBSF) (Boehringer Mannheim) for 2 h at 37ºC. The plugs were rinsed five times with TE buffer at room temperature and stored at 4ºC until use.

PFGE was performed by contour-clamped homogeneous electric field electrophoresis (CHEF) (13) using a CHEF-DRIII system ~~(from~~ Bio-Rad~~)~~. 1% (w/v) MP agarose (Roche) gel in TBE buffer (45 mM Tris-base, 0.5 mM boric acid, 0.1 mM EDTA) were used at 14ºC for separation. Linearly increasing pulse times from 10 to 200 s were used during the total run time (24 h; 5.5 V/cm). Lambda Ladder PFG Marker (New England BioLabs) and *Hansenula wingei* YB-4662-VIA marker (Bio-Rad) were used as high molecular mass DNA standards.

Plasmid DNA extraction was performed with the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer specifications, followed by electrophoresis on 1% agarose gel.

R~~Detection of transcripts by R~~everse transcription PCR (RT-PCR). *P. reinekei* MT1 was grown overnight in minimal medium with 10 mM gluconate as carbon source. During exponential growth (A_{600}=0.7) the culture was induced by addition of 0.5 mM 4-methylsalicylate and further incubated for 1 h. After addition of 8 mL RNAprotect reagent (QIAGEN), total RNA was isolated from a 12 mL aliquot using the RNeasy Mini Kit (QIAGEN), according to the instructions of the manufacturer. The resulting RNA was quantified using a GeneQuant™ 1300 Spectrophotometer (GE Healthcare) and treated with TURBO Dnase Kit (Ambion) to remove any DNA contamination. The RT-PCR was

carried out using the ImProm-II Reverse Transcription System (Promega) with 1 µg of total RNA in 20 µL reaction volume. After reverse transcription, PCR amplifications were carried out using the primer pairs P01MT1/P02MT1, P1MT1/P2MT1, P3MT1/P4MT1, P5MT1/P6MT1, P7MT1/P8MT1, P9MT1/P10MT1 or P11MT1/P12MT1 (Table S2), in a 25 µL total reaction mixture containing 1 µL of ~~total~~ cDNA, 50 pmol of each primer, 50 µM of each deoxynucleoside triphosphate, 1 mM $MgCl_2$, 5 U of *Taq* DNA polymerase and 1X reaction buffer supplied by the manufacturer. The temperature program was as follows: initial denaturation at 95°C for 5 min, 30 cycles of 30 s at 95°C, 30 s at 60°C and 60 s at 72°C, with a final extension step at 72°C for 10 min. Negative control reactions were performed in the same way, excluding reverse transcriptase from the reaction mixtures.

For the detection of transcripts of *C. necator* JMP134::X cells were grown in minimal medium with 10 mM of fructose as carbon source. During exponential growth (A_{600}=0.7) the culture was supplemented with 4-methylbenzoate (0.5 mM) and incubated for 1 h. Total RNA extraction and reverse transcription were performed as described above for *P. reinekei* MT1, using the primer pairs P1J134/P2J134, P3J134/P4J134 or P5J134/P6J134 (Table S2).

Construction of deletion mutants. Deletion mutants of *mmlC, mmlD, mmlK and mmlL* genes were constructed with the previously described Flp-FTR recombination strategy (28). Briefly, PCR fragments upstream and downstream of the targeted genes (~ 700 bp) were amplified with primer~~s~~ pairs carrying restriction sites (PstI-BamHI and BamHI-Acc65I, respectively) and cloned into the PstI-Acc65I restriction site of the pEX18Ap vector, forming the pABmml plasmid series (table S1). Subsequently, a 1.8 k~~K~~b BamHI fragment from pS858 plasmid carrying a Gm^R-GFP cassette was cloned into the BamHI restriction site formed resulting in the pAGBmml plasmid series (Table S1). The resulting constructs and the suicidal plasmids used for the construction of the different mutants are listed in

table S1.

These suicide plasmids were transferred independently to *P. reinekei* MT1 by biparental mating using *E. coli* S17λpir as donor strain. The transconjugants generated by single crossover were selected on ABC medium supplemented with Gm and merodiploids were resolved by additional plating on ABC medium supplemented with 5% sucrose. Deletion of the Gm^R-GFP cassette was achieved by conjugation of the Flp-expressing pBBFLP plasmid into the resulting strains by biparental mating using *E. coli* CC118λpir as donor and selection on ABC medium containing Tc. Plasmid pBBFLP was cured by streaking strains on ABC medium supplemented with 5% sucrose. Integrity of all mutants was verified by growth on ABC medium supplemented with different antibiotics, PCR amplification and sequencing of regions flanking the deleted genes.

Complementation of the MT1Δ*mmlL* mutant. The MT1Δ*mmlL* deletion mutant was separately complemented with the *mmlL* genes from *P. reinekei* MT1 and *C. necator* JMP134. The *mmlL* gene from *P. reinekei* MT1 was amplified using primers PmZnHydXbaIF and ZnHydSacIR, which introduce XbaI and SacI restriction sites, respectively, and cloned in the SacI-XbaI restriction site of the pBS1 vector, resulting in $pBS1mmlL_{MT1}$. The *mmlL* gene from *C. necator* JMP134 was PCR amplified with primers mmlLFW and mmlLRV and cloned using the pCR8®/GW/TOPO® Cloning Kit® (Invitrogen) to form $pTOPOmmlL_{JMP134}$. Subsequently, the insert was transferred to pBS1 by recombination-based transfer of the PCR product using the Gateway LR® Clonase™ II Enzyme Mix (Invitrogen), according to the manufacturer's instructions, resulting in $pBS1mmlL_{JMP134}$. The integrity of both pBS1mmlL plasmids was confirmed by sequencing. Both plasmids were transferred independently to the MT1Δ*mmlL* deletion mutant by biparental mating using *E. coli* S17λpir as donor. Transconjugants were selected by plating on ABC medium supplemented with Gm.

Transformation of substrates and identification of metabolites. For preparation of resting cells, wild type *P. reinekei* MT1 and mutants were grown in minimal medium with salicylate (5 mM) as carbon source at 30°C and 150 rpm. During late exponential growth, cells were harvested by centrifugation and washed with 50 mM phosphate buffer (pH 7.4). Cells were suspended in the same buffer (A_{600}= 3.0) and supplemented with 1 mM 4-methylsalicylate, 10 mM glucose and trace salts (20). Cell suspensions (three replicates) were incubated at 30°C and 150 rpm. After appropriate time intervals, aliquots were centrifuged and the cell-free supernatants were analyzed by HPLC, LC/MS or ^{1}H NMR spectroscopy.

In order to verify the chemical structure of the metabolite accumulated by mutant MT1Δ*mmlL*, the metabolite was extracted after acidification to pH 3 from the cell-free supernatant (30 mL) with five times 20 mL ethyl acetate. Extracts were dried over $MgSO_4$, evaporated to dryness on a rotary evaporator and dissolved in 0.7 mL d_6-acetone. Further samples for ^{1}H NMR spectroscopy were prepared by addition of 140 µL of D_2O water to 560 µL of cell-free supernatants.

Analytical methods. HPLC was performed with a Lichrospher SC 100 RP8 reversed phase column (125 by 4.6 mm, Bischoff). Methanol-H_2O containing 0.1 % (v/v) H_3PO_4 was used as eluent at a 1 mL/min flow rate. The column effluent was monitored simultaneously at 210, 260 and 280 nm by a diode array detector (Shimadzu). Typical retention volumes were as follows: Methanol-H_2O (58:42): 4-methylsalicylate, 5.0 mL; Methanol-H_2O (10:90): 4-ML, 5.2 mL; 3-ML, 4.7 mL.

One-dimensional or two-dimensional correlation spectroscopy (COSY) ^{1}H NMR spectra were recorded at 300 K on an AVANCE DPX 300 NMR spectrometer (Bruker). The center of the suppressed water signal (δ=4.80 ppm) was used as internal reference. The concentration of the accumulated metabolites in the samples was estimated by

comparison of the average of the integrals of the resonance lines of the protons C_2/C_6 (δ=7.49 ppm) and C_3/C_5 (δ=7.84 ppm) of 4-chlorobenzoate with the integral of the resonance lines of the protons of the methyl groups of 4-methyl-3-oxoadipate (δ=1.13 ppm) and methylsuccinate (δ=1.10 ppm), respectively. 4-chlorobenzoate was added to a final concentration of 1 mM.

Construction and testing of *lacZ* reporter fusions. The presence of promoter regions was determined with *lacZ* reporter fusions in pKGWP0, a broad range vector, which was constructed as follows. The low background activity LacZ cassette and the multiple cloning site from plasmid pTZ110 (57) were amplified using pTZ110LacZFW and pTZ110LacZRV primers (Table S2) and cloned into pCR8®/GW/TOPO® (Invitrogen), yielding pTOPO-MCS-LacZ (Table S1). The LacZ cassette and the multiple cloning site~~MCS~~ sequence were transferred from pTOPO-MCS-LacZ to the gateway-compatible and broad host range pKGW vector (31) by recombination-based transfer using the Gateway LR® Clonase™ II Enzyme Mix (Invitrogen), according to the manufacturer's instructions. The integrity of the resulting pKGWP0 vector was confirmed by sequencing.

Putative promoter regions were fused to the *lacZ* reporter gene of pKGWP0 as follows. ~~A 258 bp~~ PCR product comprising the 12-269 bp region upstream of the translational start of *mmlL* gene of *P. reinekei* MT1 was amplified with primers PmHydMT1FW and PmHydMT1RV (Table S2). Similarly, a ~~366 bp~~ PCR product comprising the 12-377 bp region upstream of the translational start of *mmlC* was amplified using the primers PmACAT_FW and PmACAT_RV. The amplified fragments were blunt-end cloned into the StuI restriction site of pKGWP0 forming plasmids pm_mmlL and pm_mmlC, respectively. The LacZ fusion of the putative promoter region of the *mmlL* gene of *C. necator* JMP134 was constructed by introducing the PCR product amplified with primers PmmlLFWEcoRI and PmmlLRVBamHI into the EcoRI/BamHI site of plasmid pKGWP0, generating plasmid

pm_mmlL$_{JMP134}$. (Danilo can you please adapt this part according to MT1?). The integrity of the constructs was verified by PCR and sequencing. Plasmids harboring the putative promoter regions were transferred to *P. reinekei* MT1 and *C. necator* JMP134::X by biparental mating using E. coli S17λpir as donor strain. Transconjugants were selected in minimal medium supplemented with Sp. Reporter fusion assays were performed as previously described (36) using 0.5 mM 4-methylsalicylate, 4-methylbenzoate, 4-ML or 3-ML as inducers. Activities are expressed in Miller units and were determined after 4 h of induction.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was deposited in the DDBJ/EMBL/GenBank databases under the accession number GQ141876.

Results

P. reinekei MT1 contains 4-methylmuconolactone methylisomerase and methylmuconolactone isomerase activities.

Previous analysis had shown that *P. reinekei* MT1 degrades 5-methyl and 4-methylsalicylate exclusively via an *ortho*-cleavage route and is able to transform 4-ML into 3-ML, indicating the presence of a 4-methylmuconolactone methylisomerase (MmlI) (7). Further transformation of 3-ML by cell extracts was not observed (7). However, like in *C. necator* JMP134 (52), a muconolactone isomerase activity of 255 U/g of protein was now observed in *P. reinekei* MT1 ~~such~~ cell extracts. The N-terminal sequence of the partially purified enzyme (MLYCVEMTVSIPRRIPLDEVERIKAAXKERAID) is in 22 out of the 32 determined residues different to the sequence of the previously characterized muconolactone isomerase in *P. reinekei* MT1 (CatC of 3-oxoadipate pathway) (7). This suggests the induction of a methylmuconolactone isomerase (MmlJ) in *P. reinekei* MT1, responsible for the reversible rearrangement of the double bond of 3-ML to form 4-methyl-

3-oxoadipate enollactone. To obtain further insights into genes and proteins involved in the metabolism of methylmuconolactones in *P. reinekei* MT1, the region surrounding the *mmlJ* gene encoding the methylmuconolactone isomerase was analyzed as outlined in materials and methods.

Identification and analysis of ORFs involved in 4-methylmuconolactone degradation in *P. reinekei* MT1.

To obtain further insights into genes and proteins involved in the metabolism of methylmuconolactones in *P. reinekei* MT1, the region surrounding the *mmlJ* gene encoding the methylmuconolactone isomerase was analyzed as outlined in materials and methods.

An overall 11.6 kb region containing twelve ORFs comprising the *mmlJ* gene was retrieved. and contained twelve ORFs. Sequence comparison with the *mml* clusters present on chromosome 1 of *C. necator* JMP134 (22, 45) and on megaplasmid pHG1 of *C. necator* H16 showed the presence of seven orthologous genes probably involved in the degradation of 4-MLmethylmuconolactone (Fig. 2). The ORFs were designated *mml* by analogy with the *mml* genes of *C. necator* JMP134. The putative activities encoded by these genes are summarized in table 1.

Pulse field gel electrophoresis (PFGE) of total DNA and plasmid DNA extraction of *P. reinekei* MT1 gave no indication of the presence of plasmids in this strain, which indicates that the region harbouring these *mml* genes is located on the chromosome like in strain JMP134 and not on a plasmid like in strain H16.

Only proteins encoded by the *mmlI* and *mmlJ* genes have a proven function in *C. necator* JMP134 (49, 52). *mmlI* gene encodes a MmlI, a unique enzyme belonging to the MmlI protein family (PF09448). The predicted enzyme of *P. reinekei* MT1 is homologous only to the MmlI of *C. necator* JMP134 (70% identity) and to the predicted MmlI of *C. necator* H16

(69% identity). Phylogenetic analysis (Fig. S1) indicated that the *mmlJ* gene product of *P. reinekei* MT1 is morest closely related to the *mmlJ* gene products of *C. necator* JMP-134 and H16, but only distantly related to muconolactone isomerases encoded in 3-oxoadipate pathway gene clusters. The *mmlL* gene encodes a putative metal-dependent hydrolase, which belongs to the metallo-β -lactamase superfamily (cl00446). At the sequence level, the most closely related enzyme (only 26% identity) with proven function is the organophosphorus hydrolase (OPHC2) of *Pseudomonas pseudoalcaligenes* C2-1, which catalyzes the hydrolysis of phosphoester bonds (Fig. S2) (14). The proteins encoded by the *mmlF* and *mmlG* genes are most closely related to those encoded by *mmlFG* genes of *C. necator* JMP134 (72-% and 68-% identity, respectively) and H16 (72-% and 70-% identity, respectively) (Fig. S3). However, they also share significant sequence identity with 3-oxoadipyl CoA transferases of proven function such as the one of *P. putida* PRS2000 (68% and 65% identity, respectively), which is part of the 3-oxoadipate pathway (42). This suggests that the *mmlF* and *mmlG* gene products have 3-oxoadipyl-CoA transferase activity and act on 4-methyl-3-oxoadipate forming 4-methyl-3-oxoadipyl-CoA by analogy with the 3-oxoadipate pathway. *mmlH* gene encodes a putative transporter of the major facilitator superfamily (cd06174), which could be responsible of internalization of extracellular muconolactones and *mmlR* encodes a putative LysR-type transcriptional regulator.

The organization of these seven genes in both *C. necator* strains and in *P. reinekei* MT1 is remarkably similar, except that in *P. reinekei* MT1 an ORF termed *mmlD* is located between the *mmlG* and *mmlH* genes. The *mmlD* gene encodes a putative acyl-CoA thioesterase which has up to 31% identity to TesB proteins, such as those from *P. putida* KT2440 (15) or *E. coli* K-12 (38), which have been described to catalyze the cleavage of C_6-C_{18} carbon fatty acids CoA thioesters and of short acyl-CoA compounds (Fig. S4).

The regions upstream of *mmlL* and downstream of *mmlJ* in *P. reinekei* MT1 differ significantly from those of both *C. necator* strains. Only in strain MT1, *mmlL* is preceded by an ORF termed *mmlC*, which encodes a putative protein of the thiolase family (cd00751). Members of this family catalyze the reversible thiolytic cleavage of 3-ketoacyl-CoA into acyl-CoA. Therefore, MmlC belongs to a broad protein family, which also comprises 3-oxoadipate CoA thiolases, such as the enzyme from *P. knackmussii B13* (32) with which it shares 42% of sequence identity (Fig. S5). This indicates that MmlC may function as a thiolase transforming 4-methyl-3-oxoadipyl-CoA into methylsuccinyl-CoA and acetyl-CoA. An additional ORF termed *mmlK* is located downstream of the *mmlJ* gene in *P. reinekei* MT1. This gene encodes a putative acetyl-CoA hydrolase/transferase with 36% identity to 4-hydroxybutyrate CoA transferase of *Anaerostipes caccae* (Fig. S6) (12).

Genes of the *mml* cluster form a single operon and are induced in the presence of 4-ML and 3-ML. The operonic structures of the *mml* gene clusters from *P. reinekei* MT1 and *C. necator* JMP134 were determined by RT-PCR using total RNA isolated from both strains, induced with 4-methylsalicylate or 4-methylbenzoate, respectively. The transcription of intergenic regions, considered of sufficient length to harbor a promoter, was assessed for seven regions in *P. reinekei* MT1 and for two (In M&M we name 3 primer pairs, is that right?) in *C. necator* JMP134 (Fig. 2). Amplification products were obtained for five out of seven assessed intergenic regions in *P. reinekei* MT1 and for both intergenic regions in *C. necator* JMP134. Absence of mRNA comprising the *orfX-mmlC* and *mmlK-orfY* intergenic regions of *P. reinekei* MT1 indicates that the regions defined as *mml* clusters form single operons, in both, *P. reinekei* MT1 and *C. necator* JMP134 (Fig. 2, (Mac: What do you prefere to present the RT_PCR results, a table or a figure? Fig 3 or table X).

As RT-PCR analysis suggests the presence of promoters upstream of *mmlC* in *P. reinekei* MT1 and upstream of *mmlL* in *C. necator* JMP134, *lacZ* transcriptional fusions of intergenic regions upstream of *mmlL* and *mmlC* in strain MT1 and of the intergenic region upstream of *mmlL* in strain JMP134 were constructed and provided in *trans* to *P. reinekei* MT1 or *C. necator* JMP134::X. β -Galactosidase assays ~~using *P. reinekei* MT1 or *C. necator* JMP134::X, where transcriptional fusions of the putative promoter regions were provided in *trans,*~~ showed an approximately 10-20 fold increase in LacZ activity after incubation with 4-methylsalicylate (tested only in *P. reinekei* MT1), 4-methylbenzoate (tested only in *C. necator* JMP134), 4-ML or 3-ML (Table 23), which indicates the functionality of all three putative promoters in their native background. ~~Most probably, 4-methylsalicylate in case of strain MT1 or 4-methylbenzoate in case of strain JMP134 do not act as inducers themselves, but intermediates such as~~ 4-ML, or 3-ML produced during metabolism of 4-methylsalicylate, in case of strain MT1, or 4-methylbenzoate in case of strain JMP134, act as true inducers~~these compounds in the respective background strain~~.

***mmlL*, *mmlC* and *mmlK* genes are essential for growth of *P. reinekei* MT1 on 4-methylsalicylate.** Directed deletions of *mmlL*, *mmlC*, *mmlD* and *mmlK* from *P. reinekei* MT1 were performed in order to clarify the role of these genes in the degradation of 4-ML. The MT1Δ*mmlL,* MT1Δ*mmlC* and MT1Δ*mmlK* mutants were unable to grow on 4-methylsalicylate as the only carbon source, whereas growth on salicylate was not affected. On the contrary, deletion of *mmlD* had no effect on the ability of strain MT1 to grow on 4-methylsalicylate. Both, wild type and mutant MT1Δ*mmlD* grew with doubling times of 1.34 ± 0.03 h and 1.25 ± 0.08 h, respectively on 4-methylsalicylate and of 1.29 ± 0.08 h and 1.23 ± 0.18 h on salicylate.

Transcomplementation of mutant MT1Δ*mmlL* with plasmids pBS1mmlL$_{MT1}$ and

pBS1mmlL$_{JMP134}$, which harbour the *mmlL* genes of *P. reinekei* MT1 and *C. necator* JMP134 respectively, fully restored the ability to grow on 4-methylsalicylate.

4-Methyl-3-oxoadipate and methylsuccinate are intermediates in the degradation of 4-ML by *P. reinekei* MT1. In order to determine ~~which are the~~if some intermediates accumulated by the MT1Δ*mmlL*, MT1Δ*mmlC* and MT1Δ*mmlK* mutants, resting cell assays were performed using 1 mM 4-methylsalicylate as substrate. HPLC and ^{1}H NMR analysis revealed that mutant MT1Δ*mmlL* transforms 4-methylsalicylate quantitatively into 3-ML, which accumulated after 24 h up to 1.13 ± 0.08 mM (Table 3). Mutants MT1Δ*mmlC* and MT1Δ*mmlK* transform 4-methylsalicylate without accumulation of UV absorbing metabolites. Analysis by ^{1}H NMR spectroscopy of cell-free supernatants after complete transformation of the substrate (6 h) as well as after extended incubation (24 h) showed that MT1Δ*mmlC* accumulates a single metabolite, the ^{1}H NMR spectrum of which was essentially identical to that previously described for the dimethylester of 4-methyl-3-oxoadipate (46). Spiking with 4-chlorobenzoate as internal standard showed that 4-methyl-3-oxoadipate accumulates stoichiometrically (1.18 ± 0.02 mM). 4-Methyl-3-oxoadipate was also excreted by the wild-type strain, however the amount accumulated did not exceed 0.23 ± 0.03 mM. Mutant MT1Δ*mmlK* accumulates two metabolites. ^{1}H NMR analysis showed that one of them also corresponds to 4-methyl-3-oxoadipate (0.66 ± 0.03 mM). A second metabolite observed in high amounts (0.49 ± 0.04 mM) was identified as methylsuccinate by comparison of its ^{1}H NMR spectral characteristics with that of authentic methylsuccinate (Table 3). Minor signals corresponding to an unidentified compound were also present.

Discussion

P. reinekei MT1 is the only natural isolate reported thus far to grow on methyl-aromatics exclusively via an *ortho*-cleavage pathway. To achieve this, *P. reinekei* MT1 harbors extraordinary catabolic features. This bacterium contains, besides an *ortho*-cleavage pathway for catechol degradation via the 3-oxoadipate pathway, a catechol 1,2-dioxygenase and a muconate cycloisomerase, which are highly specialized in the transformation of methyl-substituted substrates. The genes encoding these two enzymes are organized in one gene cluster, termed *sal* cluster, which also comprises a gene encoding salicylate 1-hydroxylase (7). This organization ensures efficient transformation of 4-methyl- and 5-methylsalicylate to 4-ML. Further degradation of 4-ML is initiated by MmlI. This enzyme is encoded by the *mml* cluster, which comprises ten catabolic genes and is transcribed as a single operon, with 4-ML, 3-ML and/or metabolites further downstream acting as inducers.

It has been previously proposed (46) that the degradation of 3-ML in *C. necator* proceeds via a route analogous to the 3-oxoadipate pathway with MmlJ, as the enzyme responsible for rearrangement of the double bond to form 4-methyl-3–oxoadipate-enollactone and thus preparing the substrate for subsequent hydrolysis (Fig. 1). However, evidence for an enzyme performing an equivalent hydrolysis of a methylsubstituted 3–oxoadipate-enollactone has not been reported thus far. The accumulation of 3-ML in mutant MT1Δ*mmlL* indicates that the *mmlL* gene product most probably is involved in the hydrolysis of the lactone ring and, therefore, that *mmlL* encodes a methyl-enollactone hydrolase, which is able to transform 4-methyl-3-oxoadipate-enollactone into 4-methyl-3-oxoadipate. The accumulation of 3-ML rather than 4-methyl-3-oxoadipate-enollactone is explained by the reversibility of the MmlJ catalyzed reaction, where the equilibrium favors the formation of the muconolactone (Fig. 1) (41).

4-Methyl-3-oxoadipate may be further metabolized by reactions identified from the

classical 3-oxoadipate pathway, where 3-oxoadipate is transformed to 3-oxoadipyl-CoA by two-component 3-oxoadipate:succinyl-CoA transferases (termed PcaIJ or CatIJ) ~~to 3-oxoadipyl-CoA~~. Due to high sequence identity with functionally characterized 3-oxoadipate:succinly-CoA transferases (32, 42), it is reasonable to assume that the *mmlFG* gene products are responsible for transformation of 4-methyl-3-oxoadipate into 4-methyl-3-oxoadipyl-CoA. Knock-out mutants of *mmlF* and *mmlG* were , ~~however,~~ not generated, as *pcaIJ* genes, which could eventually be recruited and thus mask the *mmlFG* mutant phenotype, are typically observed in *Pseudomonas* strains.

The further transformation of 3-oxoadipyl-CoA via the 3-oxoadipate pathway is catalyzed by 3-oxoadipyl-CoA thiolase forming succinyl-CoA and acetyl-CoA. 3-Oxoadipyl-CoA thiolases have been biochemically characterized from various gram-negative bacteria, including the 3-oxoadipyl-CoA thiolases of *P. knackmussii* B13 (32) or *P. putida* PRS2000 (42). Thus far, 3-oxoadipyl-CoA thiolase of gram-positive organisms have not been characterized ~~in detail~~, however genome sequencing projects show the presence of orthologous genes located in protocatechuate catabolic gene clusters of *Rhodococci* such as *R. jostii* RHA1 and whose functionality has been supported by transcriptomic and proteomic analysis (43). The close phylogenetic relation of MmlC with PcaF of *Rhodococci* (Fig. S1) and the accumulation of 4-methyl-3-oxoadipate by mutant MT1Δ*mmlC* supports the notion that this enzyme functions as 4-methyl-3-oxoadipyl-CoA thiolase transforming its substrate into methylsuccinyl-CoA and acetyl-CoA (Fig. 1~~3~~) (26). If the accumulation of 4-methyl-3-oxoadipate instead of the CoA thioester is due to the action of a thioesterase such as MmlD remains to be elucidated. However, the release to the culture medium of the free acids rather than of the CoA derivatives has been reported frequently (2, 24) and has been suggested as a general strategy of bacterial cells to prevent the depletion of the intracellular pool of CoA (40).

As stated above, methylsuccinyl-CoA may be formed by MmlC during the degradation of 4-ML. In fact, methylsuccinate is accumulated by mutant MT1Δ*mmlK* suggesting that methylsuccinate and/or its CoA derivative are metabolites of 4-ML degradation. Information on the metabolic fate of methylsuccinate or methylsuccinyl-CoA is rather scarce. Both compounds have been shown as intermediates in the metabolism of 4-methylcatechol by the fungus *Trichosporon cutaneum* (51). In this organism, 4-methylcatechol is degraded via intradiol cleavage, but in contrast to bacteria, cycloisomerization of 3-methyl-*cis,cis*-muconate produces directly 3-ML, thus circumventing the formation of 4-ML. The further metabolism occurs, as indicated above for *P. reinekei* MT1, through 4-methyl-3-oxoadipate, 4-methyl-3-oxoadipyl-CoA and methylsuccinate. Unfortunately, no sequence information is available either for genes or proteins involved in this process (50, 51). The ~~further~~ metabolism of methylsuccinyl-CoA proceeds via hydrolysis to the free acid and further reactions are assumed to occur after esterification at the C_4 carbon via itaconyl-CoA and citramalyl-CoA. Methylsuccinyl-CoA has been additionally reported as an intermediate of two cycles, the ethylmalonyl-CoA pathway for acetate assimilation in *Rhodobacter sphaeroides* (1, 23) and the glyoxylate regeneration cycle of *Methylobacterium extorquens* (34), with in both cases methylsuccinate esterified at the C_1 carbon as intermediate. In the light of these observations, the metabolic fate of methylsuccinate in *P. reinekei* MT1 and whether *mmlK* encodes a methylsuccinyl-CoA hydrolase remains to be elucidated. A significant mechanistic difference between the 3-oxoadipate pathway and the 4-ML degradative pathway has also to be considered for futurer~~ther~~ analysis. In the 3-oxoadipate activation/fission process, typically each molecule of succinyl-CoA used in activation is regenerated as soon as 3-oxoadipyl-CoA is cleaved. However, if methylsuccinyl-CoA is directly used by MmlC for thiolytic cleavage of 4-methyl-3-oxoadipy-CoA or if succinyl-CoA is independently generated and if MmlK encodes a CoA

transferase involved in such generation remains unknown. Biochemical characterization of enzymes encoded by the *mml* cluster is currently been performed, in order to characterize their substrate and cofactor specificities.

In contrast to the *mmlL*, *mmlC* and *mmlK* genes, the *mmlD* gene, which encodes a putative acyl-thioesterase, is dispensable for growth of *P. reinekei* MT1 on 4-methylsalicylate. It should be noted that not only *mmlD* gene, but also *mmlC* and *mmlK* genes are absent from the *mml* clusters of *C. necator* JMP134 and H16. As *C. necator* JMP134 has been reported to grow on 4-ML (46), the required genetic elements and their respective activities should be recruited from elsewhere on the chromosome(s). Even though 3-oxoadipyl-CoA thiolase from the 3-oxoadipate pathway obviously is not recruited to substitute for MmlC in *P. reinekei* MT1, it cannot be excluded that this happens in *C. necator*. A genome-wide analysis of both *Cupriavidus* strains indicated that only the genome of strain H16 encodes a thiolase with high sequence identity to MmlC (YP_840888, 64% identity). Interestingly, the gene encoding thisthis enzyme is preceded by another gene (YP_840887) which, the putative gene product of which exhibits significant sequence identity (55%) with MmlK. The morest closely related MmlC homologuess in *C. necator* JMP134 areare ReutA_1348 (YP_295562, 42% identity), which based on its sequence identity and the genomic context can be assumed to be involved in polyhydroxyalkanoate formation, and ReutA_1355 (YP_295567, 43% identity). If these or other unrelated proteins carry out thiolytic cleavage of 4-methyl-3-oxoadipyl-CoA in *C. necator* JMP134 remains to be elucidated.

As mentioned above an MmlK homologue is present in *C. necator* H16, but not in *C. necator* JMP134, which suggests that the channeling of methylsuccinly-CoA/methylsuccinate into the central metabolism proceeds by different pathways in *P. reinekei* MT1 and *C. necator* JMP134.

However, even though the *mml* clusters differ in the presence of *mmlC*, *mmlK* and *mmlD* genes, the organization is otherwise identical, with promoters being localized upstream of *mmlL*. It thus may be speculated~~,~~ that in order to be capable of functioning ~~in *P. reinekei* MT1~~ an archaetype *mml* gene cluster was complemented by additional genes in *P. reinekei* MT1~~in order to be capable of functioning in *P. reinekei* MT1~~. Nevertheless, it should be also noted~~,~~ that proteins encoded by homologous genes share only 65-70% of sequence identity, and as an example, the level of identity between methylmuconolactone isomerases (65%) resembles that between muconolactone isomerases from *Pseudomonas* and *Cupriavidus* strains (54-59%) rather than between muconolactone isomerases from different *Pseudomonas* strains (>80%). It can thus be assumed that both gene clusters diverged from a common ancestor in ancient times.

Despite the huge amount of information available in genome projects, an *mml* cluster with MmlI as key enzyme has been observed only in *P. reinekei* MT1, *C. necator* JMP134 and *C. necator* H16. It should ~~however,~~ be stated, however, that currently available genomes only give a highly biased overview on bacterial metabolic properties. Taking into account the widespread distribution of the 3-oxoadipate pathway at least in Proteobacteria, plus the fact that catechol 1,2-dioxygenases and muconate cycloisomerases in general exhibit significant activity with methylsubstituted substrate analogues (7, 56, 59), it can be reasoned that in the environment a significant amount of methyl substituted aromatics are funneled into a such route and that methylmuconolactone degraders could play an important role in further funneling these intermediates into the Krebs cycle.

Table 1. Open reading frames (ORFs) and genes of the *mml* gene cluster of *P. reinekei* MT1 and surrounding regions.

Gene	Gene product (aa)	Putative function of gene product	Related gene products [a]			
			Name/size (aa)	Organism	%aa identity	Accession nº (reference)
orfX	277	Itaconyl-CoA hydratase	(275)	*P. aeruginosa* PAO1	63	NP_249569
			(278)	*Pseudomonas* sp. L1	53	AAX86477
mmlC	398	Thiolase	(398)	*B. cenocepacia* MC0-3	67	ACA96001
			PaaE (401)	*P. fluorescens*	45	ABF82237 (19)
mmlL	297	Hydrolase	(296)	*C. necator* H16	79	AAP86139
			OPHC2 (324)	*P. pseudoalcaligenes*	26	CAE53631 (14)
mmlR	300	Transcriptional regulator, LysR-Type	(304)	*C. necator* H16	71	AAP86138
mmlF	230	α-subunit 3-oxoadipyl-CoA transferase	(232)	*C. necator* H16	72	AAP86137
			PcaI (231)	*P. putida* PRS2000	68	AAA25922 (42)
mmlG	222	ß-subunit 3-oxoadipyl-CoA transferase	(220)	*C. necator* H16	70	AAP86136
			PcaJ (218)	*A. baylyi* ADP1	59	AAC37147 (25)
mmlD	295	Acyl-CoA thioesterase	(303)	*M. silvestris* BL2	32	ACK50807
			ORF290 (290)	*S. viridochromogenes* DSM32 40736		AAU00099 (4)
mmlH	429	Muconolactone transporter	(428)	*C. necator* JMP134	68	AAZ60871
			MucK (426)	*A. baylyi* ADP1	30%	AAC27117 (61)
mmlI	107	4-Methylmuconolactone methylisomerase	MmlI (113)	*C. necator* JMP134	70	AAZ60870 (49)
mmlJ	92	Methylmuconolactone isomerase	(91)	*C. necator* H16	71	AAP86133
			MmlJ (91)	*C. necator* JMP134	65	AAZ60869 (53)
mmlK	422	Acetyl-CoA hydrolase /transferase	(430)	*Burkholderia* sp. H160	58	EEA04061
			(442)	*A. caccae* L1-92	36	ABA39275 (12)
orfY	320	Transcriptional regulator LysR type.	(311)	*B. glumae* BGR1	44	YP_002907790
			CnmA (310)	*P. putida* JLR11	35	AAW80266 (6)

[a] The gene product with the highest aminoacid sequence identity as well as the most closely related gene product of validated function are given.

Table 2. β -galactosidase activity resulting from expression of promoter fusions in *P. reinekei* MT1 and *C. necator* JMP134.

Inducer	LacZ activity (Miller uUnits)		
	MT1 pm_mmlC	MT1 pm_mmlL	JMP134::X p9
-	90 ± 15	310 ± 70	3.0 ± 0.2
4-methylbenzoate	ND	ND	62 ± 8
4-methylsalicylate	940 ± 160	2140 ± 80	ND
4-ML	1000 ± 50	2280 ± 70	52 ± 3
3-ML	980 ± 100	2270 ± 130	58 ± 8

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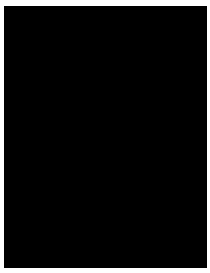
ND: not determined.

The inducers were added at the beginning of the exponential phase to a final concentration of 0.5 mM and activity was determined after a period of 4 hhours.

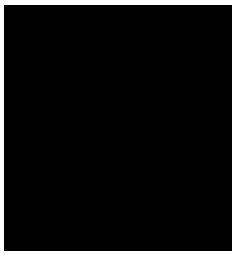
Table 3. ^{1}H NMR data of metabolites formed by ~~deletion mutants of~~ *P. reinekei* MT1 mutants.

A) ^{1}H NMR data of 3-methylmuconolactone



Nucleus	Splitting	Chemical shift (ppm)	Coupling constant (Hz)
H_1	Q	2.54 (2.57)	J_{12} = 16.4 (16) J_{13} =8.5 (8)
H_2	Q	3.00 (2.85)	J_{12} = 16.4 (16) J_{23} = 3.9 (4)
H_3	M	5.28 (5.17)	J_{13}+J_{23} = 13.2 (12)
H_4	M	2.10 (2.07)	J_{45} = 1.5 (1)
H_5	M	5.86 (5.82)	J_{45} = 1.5 (1)

B) ^{1}H NMR data of 4-methyl-3-oxoadipate



Nucleus	Splitting	Chemical shift (ppm)	Coupling constant (Hz)
H_1	Q	2.18 (2.32)	J_{12} = 15.2 (17) J_{13} = 7.9 (8.5)
H_2	Q	2.56 (2.82)	J_{12} = 15.1 (17) J_{23} = 6.6 (5.5)
H_3	M	3.05 (3.13)	J_{13}+J_{23} J_{34}= 21.6 (21)
H_4	D	1.13 (1.19)	J_{34} = 7.1 (7.0)
H_5	D	3.60 (3.64)	J_{56} = 15.4 (14.5)
H_6	D	3.48 (3.59)	J_{56} = 15.4 (14.5)

C) ^{1}H NMR data of methylsuccinate

Nucleus	Splitting *	Chemical shift (ppm)	Coupling constant (Hz)
H_1	q	2.14 (2.17)	J_{12} = 14.1 (14.5) J_{13} = 9.7 (9.8)
H_2	q	2.54 (2.56)	J_{12} = 13.2 (14.3) J_{23} = 6.2 (5.3)
H_3	m	2.65 (2.66)	J_{13}+J_{23}+ J_{34}= 22.5 (22.1)
H_4	d	1.10 (1.12)	J_{34} = 6.9 (7.0)

Chemical shift and coupling constants were calculated from representative spectra obtained from supernatants of mutants MT1Δ*mmlC* (4-methyl-3-oxoadipate) or MT1Δ*mmlK* (methylsuccinate) after incubation with 4-methylsalicylate or respective and

extracts of supernatants of mutant MT1Δ*mmlL* (3-ML) dissolved in acetone-d_6.

^{1}H NMR data were recorded at 300 MHz. ^{1}H NMR data previously described (46), for 3-methylmuconolactone at 80 MHz in $CDCl_3$ ~~are given in parentheses (46)~~ (A), ~~previously described for~~ 4-methyl-3-oxoadipate dimethylester in $CDCl_3$ measured at 200 MHz ~~are given in parentheses~~ ~~(46)~~ (B) or of authentic methylsuccinate dissolved in the same medium and recorded at 600 MHz (C), are given in parentheses.

*Doublets, quartets and multiplets are abbreviated as d, q and m, respectively.

Table S1. Strains and plasmids used in this work. (Danilo, can you complete the parts in yellow?)

Bacterial strains or plasmids	Relevant genotype	Source or reference
E. coli		
JM109	e14–(McrA–) *recA1 endA1 gyrA96 thi-1 hsdR17* (*r*K– *m*K+) *supE44 relA1* Δ(*lac-proAB*) [F´ *traD36 proAB lacI*qZΔ*M15*]	Stratagene
DH5α	*fhuA2* Δ*(argF-lacZ)U169 phoA glnV44* Φ*80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
S17λpir	Tpr Smr *recA thi pro* rK^- mK^+ *RP4*:2-Tc:MuKm Tn*7* *pir*	(18)
Mach1	Δ*rec*A1398 endA1 *tonA* Φ80ΔlacM15 ΔlacX74 *hsdR*(r_{K^-} m_{K^+})	Invitrogen
CC118λpir	(*ara-leu*) *araD* *lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1* *pir*	(27)
P. reinekei		
MT1	Wild Type	(8)
MT1Δ*mmlL*	Deletion mutant with a 944 bp excision in gene *mmlL*.	This study
MT1Δ*mmlK*	Deletion mutant with a 1323 bp excision in gene *mmlK*.	This study
MT1Δ*mmlC*	Deletion mutant with a 1125 bp excision in gene *mmlC*.	This study
MT1Δ*mmlD*	Deletion mutant with a 932 bp excision in gene *mmlD*.	This study
MT1Δ*mmlL* pBS1mmlL$_{MT1}$	MT1Δ*mmlL* deletion mutant complemented with *mmlL* of *P. reinekei* MT1	This study
MT1Δ*mmlL* pBS1mmlL$_{JMP134}$	MT1Δ*mmlL* deletion mutant complemented with *mmlL* of *C. necator* JMP134	This study
C. necator		
JMP134::X	XXX	(35, 44)
Plasmids		
pEX18Ap	ApR; oriT+*sacB*+, gene replacement vector with MCS from pUC18	(28)
pPS858	Ap^R, Gm^R;blunt-ended pPS747 PstI-XbaI fragment ligated into the blunt-ended EcoRI site of pPS856. This plasmid carries a Gm^R-GFP cassette	(28)
pBBFLP	TcR, source of inducible FLP recombinase	(17, 28)
pABmmC		This study
pABmmL		This study
pABmmD		This study
pABmmK		This study
pAGBmmC	Ap^RGm^R; pEX18Ap derivative with Gm^R-GFP cassette cloned between PCR-amplified regions flanking the *mmlC* gene (690 bp upstream and 675 bp downstream)	This study
pAGBmmL	Ap^RGm^R; pEX18Ap derivative with Gm^R-GFP cassette cloned between PCR-amplified regions flanking the *mmlL* gene (690 bp upstream and 658 bp downstream)	This study
pAGBmmD	Ap^RGm^R; pEX18Ap derivative with Gm^R-GFP cassette cloned between PCR-amplified regions flanking the *mmlD* gene (678 bp upstream and 626 bp downstream)	This study
pAGBmmK	Ap^RGm^R; pEX18Ap derivative with Gm^R-GFP cassette cloned between PCR-amplified regions flanking the *mmlK* gene (717 bp upstream and 624 bp downstream)	This study
pBS1	XXX	(REF)
pBS1mmlL$_{MT1}$	Gm^R pBS1 carrying 1269 bp fragment containing the *mmlL* gene from *P. reinekei* MT1	This study
pBS1mmlL$_{JMP134}$	Gm^R; pBS1 carrying XX bp fragment containing the *mmlL* gene from *C. necator* JMP134	This study
pTZ110	XXX	(57)
pTOPO-MCS-LacZ	XXX	This study
pKGW	XXX	(31)
pKGWP0	XXX	This study
pm_mmlL	Sp^R; 258 bp PCR product comprising the 12-269 bp region upstream the translational start of *mmlL* inserted in the StuI restriction site of pKGWP0	This study
pm_mmlC	Sp^R; 366 bp PCR product comprising the 12-377 bp region upstream the translational start of *mmlC* inserted in the StuI restriction site of pKGWP0	This study
pm_mmlL$_{JMP134}$	XXX	This study

Table S2. List of primers used in this work

Primer name	Sequence (5'-3')	Description
NH3MMLIF1	5'-AUGYTNATHTGYGTNGAAATGAC-3'	Degenerated primer; screening of fosmid library
NH3MMLIR1	5'-GCYTTDATNGKYTCRTCYTCGT-3'	Degenerated primer; screening of fosmid library
NH3MMLIF3	5'-CGTCAGTATTCCCCGTGACAT-3'	Screening of fosmid library
NH3MMLIR4	5'-TRSYGYSYSRCSRSRCGCYA-3'	Degenerated primer; screening of fosmid library
NH3MMLIR7	5'-AGGTGGGGCCACTTGCCCGAC-3'	Screening of fosmid library
P01MT1	5'- GTCCCAGCCATTGGGATG -3'	RT-PCR
P02MT1	5'- ATCAGCGCGTTTTTCATTG -3'	RT-PCR
P1 MT1	5'-CATTATGACCACGCTGTTGC -3'	RT-PCR
P2 MT1	5'- TGCAGTACACCCTCCTCCAT-3'	RT-PCR
P3 MT1	5'-ACTCCGGTTACTGCATCTGG -3'	RT-PCR
P4 MT1	5'- TTGATACGGTTGCTTGTTGC-3'	RT-PCR
P5 MT1	5'-TCTGTTCCCAGGATTTCGAC -3'	RT-PCR
P6 MT1	5'- TGAGCGATCAATGCCTGAAT-3'	RT-PCR
P7 MT1	5'-TTTTTCTGAGCGAGCCTGTT -3'	RT-PCR
P8 MT1	5'- AGCTGAACATCTGCATGTCG-3'	RT-PCR
P9 MT1	5'-TGACGTCGAAGGTAACGATG -3'	RT-PCR
P10 MT1	5'-TTGAAATCATGACGCTGTGC -3'	RT-PCR
P11 MT1	5'- ATGGTCCAACGACCATTCC -3'	RT-PCR
P12 MT1	5'- CATGCGCGGTTTCAGAAC-3'	RT-PCR
P1J134	5'- CCAGCGTATCTTCGAGGTTC -3'	RT-PCR
P2J134	5'- CCAGCGTATCTTCGAGGTTC -3'	RT-PCR
P3J134	5'- ATGCCAAAGGCAGCATTC -3'	RT-PCR
P4J134	5'- TTGTTGCTGACGACGGTAAG -3'	RT-PCR
P5J134	5'- AGTGCAGTTATCCGCTGACC -3'	RT-PCR
P6J134	5'- AGGCCCCATGTAAGTGTGAG -3'	RT-PCR
16SrDNA		RT-PCR
16SrDNA		RT-PCR
KOACTAF	5'-GGAGACTGCAGCGCGCCTGCCCAACC-3'	Deletion mutant
KOACTAR	5'-GAGAGGGATCCGATGGTCTTAGCTATTGC-3'	Deletion mutant
KOACTBF	5'-GGAGAGGATCCGGCGCCTTATATCTTAGC-3'	Deletion mutant
KOACTBR3	5'-GAGAGGGTACCAAAATGGACAAAAACATTCG-3'	Deletion mutant
KOZnHydAF2	5'-GGAGAGGTACCAGCGGCGCGATGACC-3'	Deletion mutant
KOZnHydAR	5'-GAGAGGGATCCGTTTGCCGTGCTTGTAG-3'	Deletion mutant
KOZnHydBF	5'-GGAGAGGATCCTGTACCTCAACGGTTTTTG-3	Deletion mutant
KOZnHydBR	5'-GAGAGCTGCAGAATAGACGAGGGCATTCG-3'	Deletion mutant
KOACTHAF	5'-GGAGACTGCAGCATGAGCCACGAGC-3'	Deletion mutant
KOACTHAR	5'-GAGAGGGATCCCCCTGGCAATAGCGAGC-3'	Deletion mutant
KOACTHBF	5'-GGAGAGGATCCCCCGAGCGAGG-3'	Deletion mutant
KOACTHBR	5'-GAGAGGGTACCCGCGCCGCTTGC-3'	Deletion mutant
KOACATAF	5'-GGAGACTGCAGAGAAATCGAAGTCG-3'	Deletion mutant
KOACATAR	5'-GAGAGGGATCCGGAAACCATGTGCATCG-3'	Deletion mutant
KOACATBF	5'-GGAGAGGATCCTTGCCGATGGGTGTGC-3'	Deletion mutant
KOACTABR	5'-GAGAGGGTACCCGAACAGCAGCGAG-3'	Deletion mutant
PmZnHydXbaIF	5'-TGTGTCTAGACATTATGACCACGCTGTTGC-3'	Complementation
ZnHydSacIR	5'-GGAGAGAGCTCCACAGTACATTGAGTCTGCC-3'	Complementation
mmlLFW	5- AGCAGCACCATCGGACAAT -3	Complementation
mmlLRV	5- CCGCCAACTGCCTGTAAC -3	Complementation
pTZ110LacZFW	5- GAGGCCCTTTCGTCTTCAA -3	Induction assays
pTZ110LacZRV	5- GCCTGCCCGGTTATTATTA -3	Induction assays
PmHydMT1FW	5-CATTATGACCACGCTGTTGC-3	Induction assays
PmHydMT1RV	5 -TCAACGGTTTTTGTTAGTTCG-3	Induction assays
PmACAT_FW	5'-TTGAAAGTCGCGGCAATGC-3'	Induction assays
PmACAT_RV	5'-CTCAATGGGGGCAATGC-3'	Induction assays
PmmlLFWEcoRI	5- TGCAGAATTCAAGCGCATCGTCGACAC-3	Induction assays
PmmlLRVBamHI	5- ATCGGGATCCGTGCCTTGTCTCCATTGTCC-3	Induction assays

(Danilo, in the gel pictures you send me there were only 2 band in JMP134, but you used 3 primer pairs. Should I remove from the table one of the primer pairs and which one? And which is the sequence of the 16S primers you used?)

References (The format of the references is not optimal yet, I'll correct it afterwards) I

made some corrections.

1. **Alber, B. E., R. Spanheimer, C. Ebenau-Jehle, and G. Fuchs.** 2006. Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides*. Mol Microbiol **61:**297-309.
2. **Altenschmidt, U., and G. Fuchs.** 1992. Novel aerobic 2-aminobenzoate metabolism. Purification and characterization of 2-aminobenzoate-CoA ligase, localisation of the gene on a 8-kbp plasmid, and cloning and sequencing of the gene from a denitrifying Pseudomonas sp. Eur J Biochem **205:**721-7.
3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
4. **Blodgett, J. A., J. K. Zhang, and W. W. Metcalf.** 2005. Molecular cloning, sequence analysis, and heterologous expression of the phosphinothricin tripeptide biosynthetic gene cluster from Streptomyces viridochromogenes DSM 40736. Antimicrob Agents Chemother **49:**230-40.
5. **Bruce, N. C., and R. B. Cain.** 1988. b-Methylmuconolactone, a key intermediate in the dissimilation of methylaromatic compounds by a modified 3-oxoadipate pathway evolved in nocardioform actinomycetes. FEMS Microbiology Letters **50:**233 - 239.
6. **Caballero, A., A. Esteve-Nunez, G. J. Zylstra, and J. L. Ramos.** 2005. Assimilation of nitrogen from nitrite and trinitrotoluene in *Pseudomonas putida* JLR11. J Bacteriol **187:**396-399.
7. **Camara, B., P. Bielecki, F. Kaminski, V. M. dos Santos, I. Plumeier, P. Nikodem, and D. H. Pieper.** 2007. A gene cluster involved in degradation of substituted salicylates via *ortho* cleavage in *Pseudomonas* sp. strain MT1 encodes enzymes specifically adapted for transformation of 4-methylcatechol and 3-methylmuconate. J Bacteriol **189:**1664-1674.
8. **Camara, B., C. Strompl, S. Verbarg, C. Sproer, D. H. Pieper, and B. J. Tindall.** 2007. Pseudomonas reinekei sp. nov., Pseudomonas moorei sp. nov. and Pseudomonas mohnii sp. nov., novel species capable of degrading chlorosalicylates or isopimaric acid. Int J Syst Evol Microbiol **57:**923-31.
9. **Carmona, M., M. T. Zamarro, B. Blazquez, G. Durante-Rodriguez, J. F. Juarez, J. A. Valderrama, M. J. Barragan, J. L. Garcia, and E. Diaz.** 2009. Anaerobic catabolism of aromatic compounds: a genetic and genomic view. Microbiol Mol Biol Rev **73:**71-133.
10. **Catelani, D., A. Fiecchi, and E. Galli.** 1971. Dextro-gamma-carboxymethyl-gamma-methyl-delta-alpha-butenolide. A 1,2-ring-fission product of 4-methylcatechol by Pseudomonas desmolyticum. Biochem J **121:**89-92.
11. **Cha, C. J., R. B. Cain, and N. C. Bruce.** 1998. The modified beta-ketoadipate pathway in *Rhodococcus rhodochrous* N75: Enzymology of 3-methylmuconolactone metabolism. Journal of Bacteriology **180:**6668-6673.
12. **Charrier, C., G. J. Duncan, M. D. Reid, G. J. Rucklidge, D. Henderson, P. Young, V. J. Russell, R. I. Aminov, H. J. Flint, and P. Louis.** 2006. A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology **152:**179-85.
13. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:**1582-5.
14. **Chu, X. Y., N. F. Wu, M. J. Deng, J. Tian, B. Yao, and Y. L. Fan.** 2006. Expression of organophosphorus hydrolase OPHC2 in *Pichia pastoris*: purification and characterization. Protein Expr Purif **49:**9-14.

15. **Chung, A., Q. Liu, S. P. Ouyang, Q. Wu, and G. Q. Chen.** 2009. Microbial production of 3-hydroxydodecanoic acid by pha operon and fadBA knockout mutant of Pseudomonas putida KT2442 harboring tesB gene. Appl Microbiol Biotechnol **83:**513-9.
16. **Clark, J., and O. Maaloe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J Mol Biol **23:**99-112.
17. **de las Heras, A., C. A. Carreno, and V. de Lorenzo.** 2008. Stable implantation of orthogonal sensor circuits in Gram-negative bacteria for environmental release. Environ Microbiol **10:**3305-16.
18. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol **235:**386-405.
19. **Di Gennaro, P., S. Ferrara, I. Ronco, E. Galli, G. Sello, M. Papacchini, and G. Bestetti.** 2007. Styrene lower catabolic pathway in Pseudomonas fluorescens ST: identification and characterization of genes for phenylacetic acid degradation. Arch Microbiol **188:**117-25.
20. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch~~ives~~ Microbiol~~ogy~~ **99:**61 - 70.
21. **Edgar, R. C.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.
22. **Erb, R. W., K. N. Timmis, and D. H. Pieper.** 1998. Characterization of a gene cluster from *Ralstonia eutropha* JMP134 encoding metabolism of 4-methylmuconolactone. Gene **206:**53-62.
23. **Erb, T. J., J. Retey, G. Fuchs, and B. E. Alber.** 2008. Ethylmalonyl-CoA mutase from *Rhodobacter sphaeroides* defines a new subclade of coenzyme B12-dependent acyl-CoA mutases. J Biol Chem **283:**32283-93.
24. **Fuchs, G.** 2008. Anaerobic metabolism of aromatic compounds. Ann N Y Acad Sci **1125:**82-99.
25. **Hartnett, C., E. L. Neidle, K. L. Ngai, and L. N. Ornston.** 1990. DNA sequences of genes encoding Acinetobacter calcoaceticus protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence. J Bacteriol **172:**956-66.
26. **Harwood, C. S., and R. E. Parales.** 1996. The beta-ketoadipate pathway and the biology of self-identity. Ann~~ual~~ Rev~~iew of~~ Microbiol~~ogy~~ **50:**553-590.
27. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol **172:**6557-67.
28. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.
29. **Junca, H., and D. Pieper.** 2004. Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ. Microbiol. **6:**95-110.
30. **Junca, H., and D. H. Pieper.** 2004. Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ Microbiol **6:**95-110.
31. **Karimi, M., D. Inze, and A. Depicker.** 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7:**193-5.
32. **Kaschabek, S. R., B. Kuhn, D. Muller, E. Schmidt, and W. Reineke.** 2002. Degradation

of aromatics and chloroaromatics by *Pseudomonas* sp strain B13: Purification and characterization of 3-oxoadipate: succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. J~~ournal of~~ Bacteriol~~ogy~~ **184:**207-215.

33. **Knackmuss, H.-J., M. Hellwig, H. Lackner, and W. Otting.** 1976. Cometabolism of 3-methylbenzoate and methylcatechols by a 3-chlorobenzoate utilizing *Pseudomonas*: Accumulation of (+)-2,5-dihydro-4-methyl- and (+)-2,5-dihydro-2-methyl-5-oxo-furan-2-acetic acid. Eur~~opean~~ J~~ournal of~~ Appl~~ied~~ Microbiol~~ogy~~ **2:**267 - 276.
34. **Korotkova, N., M. E. Lidstrom, and L. Chistoserdova.** 2005. Identification of genes involved in the glyoxylate regeneration cycle in *Methylobacterium extorquens* AM1, including two new genes, *meaC* and *meaD*. J Bacteriol **187:**1523-6.
35. **Ledger, T., D. H. Pieper, D. P~~é~~érez-Pantoja, and B. Gonzá~~a~~lez.** 2002. Novel insights into the interplay between peripheral reactions encoded by *xyl* genes and the chlorocatechol pathway encoded by *tfd* genes for the degradation of chlorobenzoates by *Ralstonia eutropha* JMP134. Microbiology-Sgm **148:**3431-3440.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratories.
37. **Murray, K., C. J. Duggleby, J. M. S. Trepat, and P. A. Williams.** 1972. The metabolism of benzoate and methylbenzoates via the meta-cleavage by *Pseudomonas arvilla* mt-2. Eur. J. Biochem. **28:**301-310.
38. **Naggert, J., M. L. Narasimhan, L. DeVeaux, H. Cho, Z. I. Randhawa, J. E. Cronan, Jr., B. N. Green, and S. Smith.** 1991. Cloning, sequencing, and characterization of Escherichia coli thioesterase II. J Biol Chem **266:**11044-50.
39. **Nikodem, P., V. Hecht, M. Schlomann, and D. H. Pieper.** 2003. New bacterial pathway for 4-and 5-chlorosalicylate degradation via 4-chlorocatechol and maleylacetate in *Pseudomonas* sp~~ -~~strain MT1. J~~ournal of~~ Bacteriol~~ogy~~ **185:**6790-6800.
40. **Olivera, E. R., B. Minambres, B. Garcia, C. Muniz, M. A. Moreno, A. Ferrandez, E. Diaz, J. L. Garcia, and J. M. Luengo.** 1998. Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: The phenylacetyl-CoA catabolon. Proc. Natl. Acad. Sci. U. S. A. **95:**6419-6424.
41. **Ornston, L., and R. Sta~~n~~nier.** 1966. The conversion of catechol and protocatechuate to beta-ketoadipate by Pseudomonas putida. 1. Biochemistry. J Biol Chem **241:**3776-3786.
42. **Parales, R. E., and C. S. Harwood.** 1992. Characterization of the genes encoding beta-ketoadipate: succinyl-coenzyme A transferase in *Pseudomonas putida*. J~~ournal of~~ Bacteriol~~ogy~~ **174:**~~pp.~~ 4657-4666.
43. **Patrauchan, M. A., C. Florizone, M. Dosanjh, W. W. Mohn, J. Davies, and L. D. Eltis.** 2005. Catabolism of benzoate and phthalate in *Rhodococcus* sp. strain RHA1: redundancies and convergence. J Bacteriol **187:**4050-63.
44. **Pemberton, J. M., B. Corney, and R. H. Don.** Evolution and spread of pesticides degrading ability among soil micro-organisms.pp. 287-299.
45. **P~~é~~érez-Pantoja, D., R. De la Iglesia, D. H. Pieper, and B. Gonzá~~a~~lez.** 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. FEMS Microbiol Rev **32:**736-94.
46. **Pieper, D. H., K.-H. Engesser, R. H. Don, K. N. Timmis, and H.-J. Knackmuss.** 1985. Modified ortho-cleavage pathway in *Alcaligenes eutrophus* JMP134 for the degradation of 4-methylcatechol. FEMS Microbiol~~ogy~~ Lett~~ers~~ **29:**63 - 67.
47. **Pieper, D. H., K.-H. Engesser, and H.-J. Knackmuss.** 1989. Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP 134. Arch~~ives of~~ Microbiol~~ogy~~ **151:**365-371.
48. **Pieper, D. H., H.-J. Knackmuss, and K. N. Timmis.** 1993. Accumulation of 2-chloromuconate during metabolism of 3-chlorobenzoate by *Alcaligenes eutrophus* JMP134.

Applied Microbiology and Biotechnology **39:**563 - 567.

49. **Pieper, D. H., K. Stadler-Fritzsche, H. J. Knackmuss, K. H. Engesser, N. C. Bruce, and R. B. Cain.** 1990. Purification and characterization of 4-methylmuconolactone methylisomerase, a novel enzyme of the modified 3-oxoadipate pathway in the gram-negative bacterium *Alcaligenes eutrophus* JMP134. Biochem J **271:**529-34.
50. **Powlowski, J. B., and S. Dagley.** 1985. b-Ketoadipate pathway in *Trichosporon cutaneum* modified for methyl-substituted metabolites. Journal of Bacteriology **163:**1126 - 1135.
51. **Powlowski, J. B., J. Ingebrand, and S. Dagley.** 1985. Enzymology of the b-ketoadipate pathway in *Trichosporon cutaneum*. Journal of Bacteriology **163:**1136 - 1141.
52. **Prucha, M., A. Peterseim, and D. H. Pieper.** 1997. Evidence for an isomeric muconolactone isomerase involved in the metabolism of 4-methylmuconolactone by *Alcaligenes eutrophus* JMP134. Arch Microbiol **168:**33-8.
53. **Prucha, M., A. Peterseim, K. N. Timmis, and D. H. Pieper.** 1996. Muconolactone isomerase of the 3-oxoadipate pathway catalyzes dechlorination of 5-chloro-substituted muconolactones. European Journal of Biochemistry **237:**350-356.
54. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406-25.
55. **Sala-Trepat, J. M., K. Murray, and P. A. Williams.** 1972. The metabolic divergence in the meta-cleavage of catechols by Pseudomonas putida NCIB 10015. Eur. J. Biochem. **28:**347-356.
56. **Schmidt, E., and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds

Conversion of chlorinated muconic acids into maleoylacetic acid. Biochemical Journal **192:**339 - 347.

57. **Schweizer, H. P., and R. Chuanchuen??.** 2001. Small broad-host-range lacZ operon fusion vector with low background activity. Biotechniques **31:**1258, 1260, 1262.
58. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:**1596-9.
59. **Vollmer, M. D., H. Hoier, H. J. Hecht, U. Schell, J. Groning, A. Goldman, and M. Schlomann.** 1998. Substrate specificity of and product formation by muconate cycloisomerases: an analysis of wild-type enzymes and engineered variants. Appl. Environ. Microbiol. **64:**3290-3299.
60. **Watanabe, K.** 2001. Microorganisms relevant to bioremediation. Curr. Opin. Biotechnol. **12:**237-241.
61. **Williams, P. A., and L. E. Shaw.** 1997. mucK, a gene in *Acinetobacter calcoaceticus* ADP1 (BD413), encodes the ability to grow on exogenous cis,cis-muconate as the sole carbon source. Journal of Bacteriology **179:**5935-5942.

Abbreviate the journal name in several refs

Ref 29 and 30 are repeated OJO