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Modified 3-Oxoadipate Pathway for the Biodegradation of Methylaromatics in *Pseudomonas reinekei* MT1▽†

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Catechols are central intermediates in the metabolism of aromatic compounds. Degradation of 4-methylcatechol via 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 *Pseudomonas 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. Promoter fusion assays identified 4-methyl-3-oxoadipate as an inducer. 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 a loss of the capability of the strain MT1 to open the lactone ring, suggesting its function as a 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-coenzyme A (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 an *mmlK* deletion mutant indicates that the encoded acetyl-CoA hydrolase/transferase is crucial for channeling methylsuccinate into the central metabolism.

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 (27, 61). An extensive array of substituted aromatic structures are transformed to a few central intermediates that undergo ring cleavage (10, 29).

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 (29). 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 (29). 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 an enol-lactone hydrolase, and the resulting 3-oxoadipate is in turn channeled by 3-oxoadipate:succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase into 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) accumulates (11, 35), since muconolactone isomerases require a proton at the C-4 carbon atom to catalyze the isomerization to enol-lactone (13). Most bacteria described so far mineralize methylaromatics via the alternative *meta* (extradiol) cleavage pathway (39, 56).

Only two bacteria (*Cupriavidus necator* JMP134 [47] and *Rhodococcus rhodochrous* N75 [5]) have been reported to degrade 4-methylcatechol via an *ortho* cleavage pathway and to be capable of 4-ML mineralization. *C. necator* JMP134 harbors the *mml* gene cluster (CP000090: ReutA1502 to ReutA1508), which has been proposed to consist of seven open reading frames (ORFs) encoding enzymes and putative proteins involved in the metabolism of 4-ML (24, 46). Only 4-methylmuconolactone methylisomerase (MmlI) and methymuconolactone isomerase (MmlJ), encoded by the *mmlI* and *mmlJ* genes, respectively, have a described function (50, 53). By sequence comparison with this gene cluster, *Cupriavidus necator* H16 was also found to harbor a putative *mml* gene cluster (AY305378: PHG384 to PHG390). However, whether this cluster is functional or not remains to be elucidated.

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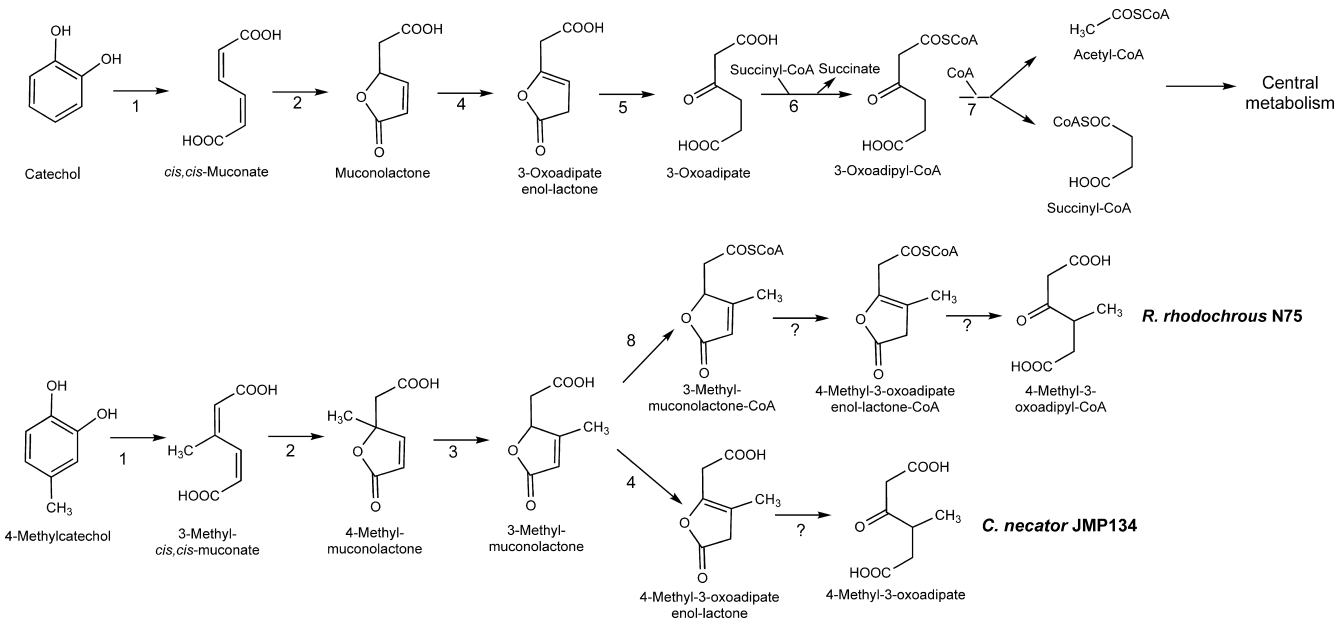


FIG. 1. Present status of knowledge on *ortho* cleavage pathway for catechol (top) or 4-methylcatechol (bottom) degradation. Metabolic routes for 4-methylcatechol have been proposed for *C. necator* JMP134 (lower branch) and *R. rhodochrous* N75 (upper branch). Enzyme names are as follows: 1, catechol 1,2-dioxygenase; 2, muconate cycloisomerase; 3, 4-methylmuconolactone methylisomerase; 4, muconolactone or methylmuconolactone isomerase; 5, 3-oxoadipate enol-lactone hydrolase; 6, 3-oxoadipate:succinyl-CoA transferase; 7, 3-oxoadipyl-CoA thiolase; 8, 3-methylmuconolactone-CoA synthetase; ?, unknown enzymes.

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 (6, 50). 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 (53). In addition, it has been proposed that in this strain, the enol-lactone intermediate may be transformed to 4-methyl-3-oxoadipate by a hydrolase (47). However, no typical enol-lactone hydrolase activity toward methyl-substituted muconolactones has been observed as yet (53).

In contrast, in *R. rhodochrous* N75, 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 (12). 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 Cámara et al. reported that *Pseudomonas reinekei* MT1 degrades 4-methylsalicylate via *ortho* cleavage of 4-methylcatechol (8). 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 (8), which indicates that methyl-substituted 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 (48), *P. reinekei* MT1 relies solely on the *ortho* cleavage route to mineralize methylcatechols and thus represents an ideal system with which to study this pathway in detail (8).

In this report, we describe 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.

MATERIALS AND METHODS

Chemicals. 4-ML, 3-ML, and 5-chloro-3-methylmuconolactone were prepared as described earlier (35, 47, 49). Methylsuccinate was obtained from Sigma-Aldrich (Steinheim, Germany).

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *P. reinekei* strain MT1 was grown in minimal medium as previously described (41) with 5 mM salicylate or 4-methylsalicylate as the 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 (37), was grown in the same medium with 2.5 mM 4-methylbenzoate as the 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 [18] supplemented with trace metals [22] and 20 mM citrate) was used. Antibiotics were used at the following concentrations: for *E. coli*, carbenicillin (Cb) (100 μg/ml), gentamicin (Gm) (10 μg/ml), tetracycline (Tc) (10 μg/ml), and spectinomycin (Sp) (100 μg/ml); for *P. reinekei*, Gm (200 μg/ml), Tc (15 μg/ml), and Sp (100 μg/ml); and for *C. necator* JMP134, Gm (20 μg/ml), Sp (100 μg/ml), and kanamycin (Km) (100 μg/ml).

Enzymatic assays. Cell extracts of *P. reinekei* MT1 grown on 4-methylsalicylate were prepared as previously described (41). MmlI activity was measured by high-performance liquid chromatography (HPLC) following the transformation of 4-ML to 3-ML as reported previously (50). Activity of MmlJ was determined spectrophotometrically by measuring the transformation of 200 μM 5-chloro-3-

methylmuconolactone in Tris-HCl (50 mM, pH 7.5) as previously described (54). One unit (U) was defined as μmol 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 (GE Healthcare, Piscataway, NJ). Cells extracts were applied directly onto the column, and proteins were eluted by using a linear gradient of 0 to 0.5 M NaCl over 33 ml at a flow rate of 0.5 ml/min. MmlJ eluted at 0.37 ± 0.01 M NaCl. Aliquots of highly active fractions were subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane, and major protein bands with a molecular mass of ~10 kDa were analyzed by N-terminal sequencing (32).

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 the degenerate primers NH3MMLIF1 and NH3MMLIR1, which were designed based on the N-terminal protein sequence of the partially purified MmlJ protein from *P. reinekei* MT1. Primer sequences are shown in Table S2 in the supplemental material. The 75-bp PCR product generated was cloned into the pGEM-T Easy vector (Promega, Madison, WI), transformed into *E. coli* Max Efficiency DH5α competent cells (Invitrogen, Carlsbad, CA), and sequenced. Based on the cloned sequence, a specific forward primer, NH3MMLIF3, was designed and used in a second PCR round with a reverse degenerate 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, La Jolla, CA), and sequenced. A previously constructed fosmid library of *P. reinekei* MT1 genomic DNA (8) was screened by PCR using the primers NH3MMLIF3 and NH3MMLIR7, specific for the *mmlJ* gene. Positive fosmid clones were purified using the FosmidMAX DNA purification kit (Epicentre, Madison, WI) and subjected to direct sequencing of upstream and downstream regions of the *mmlJ* gene, using the ABI Prism BigDye Terminator v1.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA) 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 the BLASTX and BLASTP programs from the NCBI website (3). Translated protein sequences were aligned with the MUSCLE software program, using default values (23). Phylogenetic trees were constructed using the MEGA4 software program (59), using the neighbor-joining algorithm (55) with *p*-distance correction and pairwise deletion of gaps and missing data. A total of 100 bootstrap replications were performed to test for branch robustness.

Extrachromosomal DNA extraction. Detection of megaplasmids was attempted by pulsed-field gel electrophoresis (PFGE). *P. reinekei* MT1 was cultivated at 30°C in 100 ml LB medium to an A_{600} of 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% (wt/vol) low-melting-point agarose (Invitrogen). The mixture was poured into plugs, which were incubated overnight at 50°C 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 [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF)] (Boehringer Mannheim, Mannheim, Germany) for 2 h at 37°C. The plugs were rinsed five times with TE buffer at room temperature and stored at 4°C until used.

PFGE was performed by contour-clamped homogeneous electric field electrophoresis (CHEF) (15) using a CHEF-DRIII system (Bio-Rad, München, Germany). Multipurpose agarose (1% [wt/vol]) (Roche, Berlin, Germany) gel in TBE buffer (45 mM Tris-base, 0.5 mM boric acid, 0.1 mM EDTA) was used at 14°C 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 pulsed-field gel marker (New England BioLabs, Ipswich, MA) and *Hansenula wingei* YB-4662-VIA marker (Bio-Rad) were used as high-molecular-mass DNA standards.

Plasmid DNA extraction was performed using the QIAprep spin miniprep kit (Qiagen, Chatsworth, CA) according to the manufacturer's specifications, followed by electrophoresis on 1% agarose gels.

RT-PCR. *P. reinekei* MT1 was grown overnight in minimal medium with 10 mM gluconate as a 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 minikit (Qiagen), according to the manufacterer's instructions. The resulting RNA was quantified using a GeneQuant 1300 spectrophotometer (GE Healthcare) and treated with the Turbo DNase kit (Ambion, Austin, TX) to remove any DNA contamination. The reverse transcription-PCR (RT-PCR) was carried out using the ImProm-II reverse transcription system (Promega) with 1 μg of total RNA in a 20-μl reaction volume. After reverse transcription, PCR amplifications were carried out using the primer pair P01MT1/P02MT1, P1MT1/P2MT1, P3MT1/P4MT1, P5MT1/P6MT1, P7MT1/P8MT1, P9MT1/P10MT1, or P11MT1/P12MT1 (see Table S2 in the supplemental material) in a 25-μl total reaction mixture containing 1 μl of cDNA, 50 pmol of each primer, 50 μM (each) deoxynucleoside triphosphates, 1 mM $MgCl_2$, 5 U of *Taq* DNA polymerase, and 1× reaction buffer supplied by the manufacturer. The temperature program was as follows: initial denaturation at 95°C for 5 min, and 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 carried out 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 fructose as a 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 pair P1J134/P2J134 or P3J134/P4J134.

Amplification products (5 μl) were separated on 1% agarose gels after mixing with 1 μl of SYBR Safe DNA gel stain (Invitrogen).

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 (58) were amplified using the pTZ110LacZFW and pTZ110LacZRV primers and cloned into pCR8/GW/TOPO (Invitrogen) to yield pTOPO-MCS-LacZ. The LacZ cassette and the multiple cloning site sequence were transferred from pTOPO-MCS-LacZ to the gateway-compatible and broad-host-range pKGW vector (33) 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 bp 12 to 269 region upstream of the translational start site of the *mmlL* gene of *P. reinekei* MT1 was amplified with the primers PmHydMT1FW and PmHydMT1RV. Similarly, a 366-bp PCR product comprising the bp 12 to 377 region upstream of the translational start site 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 the 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 a 332-bp PCR product comprising the sequence immediately upstream of the translational start site of *mmlL*. This region was amplified with the primers PmmlLFWEcoRI and PmmlLRVBamHI for insertion into the EcoRI/BamHI site of plasmid pTZ110 to generate plasmid pTZpm_mmlL_{JMP134}. The cassette containing *lacZ* fused to the upstream region of *mmlL* from plasmid pTZpm_mmlL_{JMP134} was amplified using the pTZ110LacZFW and pTZ110LacZRV primers and cloned into pCR8/GW/TOPO (Invitrogen) to yield pTOPO-pm_mmlL_{JMP134}-lacZ. This cassette was transferred from pTOPO-pm_mmlL_{JMP134}-lacZ to the pKGW vector by recombination-based transfer using the Gateway LR Clonase II enzyme mix to give pm_mmlL_{JMP134}. The integrity of the constructs was verified by PCR and sequencing. Plasmids harboring the putative promoter regions were transferred to *P. reinekei* MT1, *P. reinekei* MT1Δ*mmlL*, *P. reinekei* MT1Δ*mmlC*, and *C. necator* JMP134::X by biparental mating using *E. coli* S17λpir as a donor strain. Transconjugants were selected in minimal medium supplemented with Sp. Reporter fusion assays were performed as previously described (38) using 0.5 mM 4-methylsalicylate, 4-methylbenzoate, 4-ML, or 3-ML as an inducer. Activities are expressed in Miller units and were determined after 4 h of induction.

Construction of deletion mutants. *mmlC*, *mmlD*, *mmlK*, and *mmlL* gene deletion mutants were constructed with the previously described Flp-Flp recombinase target (FRT) recombination strategy (31). Briefly, PCR fragments upstream and downstream of the targeted genes (~700 bp) were amplified with primer 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. Subsequently, a 1.8-kb BamHI fragment from the pS858 plasmid carrying a Gm^r-green fluorescent protein (GFP) cassette was cloned into the BamHI restriction site formed to give the pAGBmml plasmid series. The resulting constructs and the suicidal plasmids used for the construction of the different mutants are listed in Table S1 in the supplemental material.

These suicide plasmids were transferred independently into *P. reinekei* MT1 by biparental mating using *E. coli* S17λpir as a 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 (19) into the resulting strains by biparental mating using *E. coli* CC118λpir (30) as a donor and selec-

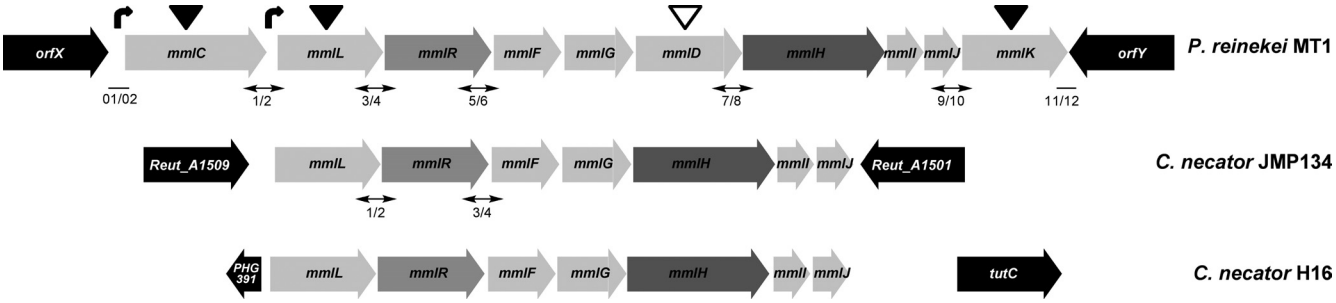


FIG. 2. Comparison of the *mml* gene cluster of *P. reinekei* MT1 with those of *C. necator* JMP134 and *C. necator* H16. Genes encoding catabolic enzymes, transcriptional regulators, and putative transporters are indicated in light gray, gray, and dark gray, respectively. Genes framing the *mml* gene clusters are indicated in black. Genes essential for growth on 4-methylsalicylate are indicated with black triangles, whereas those that are dispensable are indicated with unfilled triangles. Numbers below the arrows indicate the primer pairs utilized to assess transcription of intergenic regions. Double-headed arrows represent intergenic regions transcribed during growth on 4-methylsalicylate (MT1) or 4-methylbenzoate (JMP134::X), and lines indicate those regions not transcribed. Experimentally determined promoter regions are indicated by curved arrows.

tion on ABC medium containing Tc. Plasmid pBBFLP was cured by streaking strains on ABC medium supplemented with 5% sucrose. The 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 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 the primers PmZnHydXbaIF and ZnHydSacIR, which introduce XbaI and SacI restriction sites, respectively, and cloned into the SacI-XbaI restriction site of the pBS1 vector (4) to give pBS1mmlL$_{MT1}$. The *mmlL* gene from *C. necator* JMP134 was PCR amplified with the 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, to give pBS1mmL$_{JMP134}$. 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 (20) as a 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 a carbon source at 30°C. 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 (22). 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 and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

In order to verify the chemical structure of the metabolite accumulated by the 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, Leonberg, Germany). Methanol-H_2O containing 0.1% (vol/vol) H_3PO_4 was used an as eluent at a 1-ml/min flow rate. The column effluent was monitored simultaneously at 210, 260, and 280 nm with a diode array detector (Shimadzu, Duisburg, Germany). 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 and two-dimensional correlation spectroscopy (COSY) ^{1}H NMR spectra were recorded at 300 K on Avance DPX 300 and DMX 600 NMR spectrometers (Bruker, Bremen, Germany). The center of the suppressed water signal (δ = 4.80 ppm) was used as an internal reference. The concentrations of the accumulated metabolites in the samples were estimated by comparison of the average of the integrals of the resonance lines of the protons H_2/H_6 (δ = 7.49 ppm) and H_3/H_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) or methylsuccinate (δ = 1.10 ppm), respectively. 4-Chlorobenzoate was added to a final concentration of 1 mM.

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 analyses have 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) (8). Further transformation of 3-ML by cell extracts was not observed (8). However, analysis for muconolactone isomerase activity revealed the presence of such activity (255 U/g of protein) in *P. reinekei* MT1 cell extracts, similar to the situation in *C. necator* JMP134 (53). The N-terminal sequence of the partially purified enzyme (MLYCVEMTVSIPRRIPLD EVERIKAAXKERAID) differs from that of the previously characterized muconolactone isomerase of this strain (CatC of the 3-oxoadipate pathway) (9) in 22 out of the 32 determined residues. 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 enol-lactone.

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 containing 12 ORFs was retrieved. Sequence comparison with the *mml* clusters present on chromosome 1 of *C. necator* JMP134 (24, 46) and on megaplasmid pHG1 of *C. necator* H16 showed the presence of seven orthologous genes probably involved in the degradation of 4-ML (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.

PFGE of total DNA and plasmid DNA extraction of *P.*

TABLE 1. ORFs and genes of the *mml* gene cluster of *P. reinekei* MT1 and surrounding regions

Gene	Gene product		Related gene product[b]			
	Size (aa[a])	Putative function	Name (size [aa])	Organism	% aa identity	Accession no. (reference)
orfX	277	Itaconyl-CoA hydratase	(275)	*Pseudomonas aeruginosa* PAO1	63	NP_249569
			(278)	*Pseudomonas* sp. L1	53	AAX86477
mmlC	398	Thiolase	(398)	*Burkholderia cenocepacia* MC0-3	67	ACA96001
			PaaE (401)	*Pseudomonas fluorescens*	45	ABF82237 (21)
mmlL	297	Hydrolase	(296)	*C. necator* H16	79	AAP86139
			OPHC2 (324)	*Pseudomonas pseudoalcaligenes*	26	CAE53631 (16)
mmlR	300	Transcriptional regulator, LysR type	(304)	*C. necator* H16	71	AAP86138
mmlF	230	3-Oxoadipyl-CoA transferase α-subunit	(232)	*C. necator* H16	72	AAP86137
			PcaI (231)	*P. putida* PRS2000	68	AAA25922 (44)
mmlG	222	3-Oxoadipyl-CoA transferase β-subunit	(220)	*C. necator* H16	70	AAP86136
			PcaJ (218)	*Acinetobacter baylyi* ADP1	59	AAC37147 (28)
mmlD	295	Acyl-CoA thioesterase	(303)	*Methylocella silvestris* BL2	32	ACK50807
mmlH	429	Transporter	(428)	*C. necator* JMP134	68	AAZ60871
			MucK (426)	*A. baylyi* ADP1	30	AAC27117 (62)
mmlI	107	4-Methylmuconolactone methylisomerase	MmlI (113)	*C. necator* JMP134	70	AAZ60870 (50)
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 (14)
orfY	320	Transcriptional regulator, LysR type	(311)	*Burkholderia glumae* BGR1	44	YP_002907790
			CnmA (310)	*P. putida* JLR11	35	AAW80266 (7)

[a] aa, amino acids.
[b] The gene product with the highest amino acid sequence identity and the most closely related gene product of validated function are given.

reinekei MT1 gave no indication of the presence of plasmids in this strain, which suggests that the region harboring these *mml* genes is located on the chromosome, as in strain JMP134, and not on a plasmid as in strain H16.

Only proteins encoded by the *mmlI* and *mmlJ* genes have a proven function in *C. necator* JMP134 (50, 53). The *mmlI* gene encodes MmlI, a unique enzyme belonging to the MmlI protein family (PF09448). The predicted enzyme of *P. reinekei* MT1 shares 70% and 69% of sequence identity with MmlI of *C. necator* JMP134 and with the predicted MmlI protein of *C. necator* H16, the only homologues currently available from public databases. Phylogenetic analysis indicated that the *mmlJ* gene product of *P. reinekei* MT1 is most closely related to the *mmlJ* gene products of *C. necator* JMP134 and H16 but only distantly related to muconolactone isomerases encoded in 3-oxoadipate pathway gene clusters (see Fig. S1 in the supplemental material). 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 (see Fig. S2 in the supplemental material) (16). The proteins encoded by the *mmlF* and *mmlG* genes are most closely related to those encoded by the *mmlF* and *mmlG* genes of *C. necator* JMP134 (72% and 68% identity, respectively) and H16 (72% and 70% identity, respectively) (see Fig. S3 in the supplemental material). However, they also share significant sequence identity with 3-oxoadipyl CoA transferases of proven function, such as the one from *Pseudomonas putida* PRS2000 (68% and 65% identity, respectively), which is part of the 3-oxoadipate pathway (44). 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. The *mmlH* gene encodes a putative transporter of the major facilitator superfamily (cd06174), which could be responsible for internalization of extracellular muconolactones or dicarboxylic acids, and *mmlR* encodes a putative LysR-type transcriptional regulator.

The organization of these seven genes both in *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 (17) or *E. coli* K-12 (40), which have been described to catalyze the cleavage of C_6-C_{18} carbon fatty acid CoA thioesters and of short acyl-CoA compounds (see Fig. S4 in the supplemental material).

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 contains 3-oxoadipate CoA thiolases, such as the enzyme from *Pseudomonas knackmussii* B13 (34) with which it shares 42% sequence identity (see Fig. S5 in the supplemental material). 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-hy-

TABLE 2. β-Galactosidase activity resulting from expression of promoter fusions in *P. reinekei* MT1 and *C. necator* JMP134[a]

Strain tested	β-Galactosidase activity with inducer				
	None	4-Methylbenzoate	4-Methylsalicylate	4-ML	3-ML
MT1(pm_mmlC)	90 ± 15	ND	940 ± 160	1000 ± 50	980 ± 100
MT1(pm_mmlL$_{MT1}$)	310 ± 70	ND	2140 ± 80	2280 ± 70	2270 ± 130
JMP134::X(pm_mmlL$_{JMP134}$)	3.0 ± 0.2	62 ± 8	ND	52 ± 3	58 ± 8
MT1Δ*mmlL*(pm_mmlC)	86 ± 23	ND	ND	157 ± 38	ND
MT1Δ*mmlL*(pm_mmlL$_{MT1}$)	36 ± 4	ND	ND	38 ± 3	ND
MT1Δ*mmlC*(pm_mmlC)	32 ± 2	ND	ND	620 ± 20	ND
MT1Δ*mmlC*(pm_mmlL$_{MT1}$)	290 ± 15	ND	ND	5850 ± 290	ND

[a] 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 h. ND, not determined. Whereas *P. reinekei* MT1 and *C. necator* JMP134 are capable of mineralizing the inducers, *P. reinekei* MT1Δ*mmlL* transforms 4-ML quantitatively to 3-ML and *P. reinekei* MT1Δ*mmlC* transforms 4-ML quantitatively to 4-methyl-3-oxoadipate. Activities are expressed as Miller units.

droxybutyrate CoA transferase of *Anaerostipes caccae* (see Fig. S6 in the supplemental material) (14).

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 and 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 *C. necator* JMP134 (Fig. 2). Amplification products were obtained for five out of the seven assessed intergenic regions in *P. reinekei* MT1 and for both intergenic regions in *C. necator* JMP134 (see Fig. S7 in the supplemental material). An 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).

Since 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 showed an approximately 10- to 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 2), which indicates the functionality of all three putative promoters in their native background. To identify the nature of the inducer, transcriptional fusions of *lacZ* and intergenic regions upstream of *mmlL* and *mmlC* of strain MT1 were also introduced in *P. reinekei* MT1Δ*mmlL* and *P. reinekei* MT1Δ*mmlC*, which are incapable of mineralizing 4-methylsalicylate via 4-ML due to deletions in the *mmlL* and *mmlC* genes and quantitatively accumulate 3-ML or 4-methyl-3-oxoadipate, respectively (see below). Expression of the *lacZ* fusions was observed only in the MT1ΔmmlC background, indicating 4-methyl-3-oxoadipate is the inducer of the *mml* gene cluster.

***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. In contrast, deletion of *mmlD* had no effect on the ability of strain MT1 to grow on 4-methylsalicylate (2 mM). 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 with doubling times of 1.29 ± 0.08 h and 1.23 ± 0.18 h on salicylate.

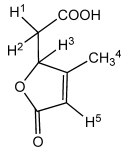
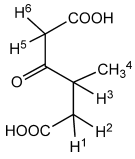
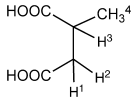
Complementation of mutant MT1Δ*mmlL* with plasmid pBS1mmlL$_{MT1}$, harboring the *mmlL* gene of *P. reinekei* MT1, was performed in order to rule out possible polar effects. Furthermore, transcomplementation with pBS1mmlL$_{JMP134}$, harboring the *mmlL* gene of *C. necator* JMP134, was performed. In both cases, the ability to grow on 4-methylsalicylate was fully restored.

4-Methyl-3-oxoadipate and methylsuccinate are intermediates in degradation of 4-ML by *P. reinekei* MT1. In order to determine the intermediates accumulated by the MT1Δ*mmlL*, MT1Δ*mmlC*, and MT1Δ*mmlK* mutants, resting cell assays were performed using 1 mM 4-methylsalicylate as a substrate. HPLC and ^{1}H NMR analysis revealed that the mutant MT1Δ*mmlL* transforms 4-methylsalicylate quantitatively into 3-ML, which accumulated after 24 h up to 1.13 ± 0.08 mM (Table 3). The 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), indicated 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 (Table 3) (47). Spiking with 4-chlorobenzoate as an 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, although the amount accumulated did not exceed 0.23 ± 0.03 mM. The mutant MT1Δ*mmlK* accumulates two metabolites. ^{1}H NMR analysis indicated that one of these corresponds to 4-methyl-3-oxoadipate (0.66 ± 0.03 mM). A second metabolite, observed in large amounts (0.49 ± 0.04 mM), was identified as methylsuccinate by comparison of its ^{1}H NMR spectral characteristics with those of authentic material (Table 3).

DISCUSSION

P. reinekei MT1 is the only natural isolate reported thus far to grow on methylaromatics exclusively via an *ortho* cleavage

TABLE 3. ^{1}H NMR data of metabolites formed by *P .reinekei* MT1 deletion mutants[a]

Metabolite	Structure	Nucleus	Splitting	Chemical shift (ppm)	Coupling constant(s) (Hz)
3-Methylmuconolactone		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)
4-Methyl-3-oxoadipate		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)
Methylsuccinate		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)

[a] Chemical shift and coupling constants were calculated from representative spectra obtained from supernatants of mutant MT1Δ*mmlC* (4-methyl-3-oxoadipate) or MT1Δ*mmlK* (methylsuccinate) after incubation with 4-methylsalicylate or extract of supernatant 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 for 3-methylmuconolactone recorded at 80 MHz in $CDCl_3$ (47) (top section) and those of 4-methyl-3-oxoadipate dimethylester in $CDCl_3$ recorded at 200 MHz (47) (middle section) or of authentic methylsuccinate dissolved in the same medium and recorded at 600 MHz (bottom section) are given in parentheses. Doublets, quartets, and multiplets are abbreviated as d, q and m, respectively.

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 for the transformation of methyl-substituted substrates (8). The genes encoding these two enzymes are organized in a gene cluster, termed the *sal* cluster, which also comprises a gene encoding salicylate 1-hydroxylase (8). 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, comprising 10 catabolic genes and transcribed as a single operon, with 4-methyl-3-oxoadipate acting as an inducer (Fig. 2).

Previously it was proposed 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 enol-lactone, thus preparing the substrate for subsequent hydrolysis (47, 53) (Fig. 1). However, evidence for an enzyme performing an equivalent hydrolysis of a methylsubstituted 3-oxoadipate enol-lactone has not been reported thus far. The accumulation of 3-ML in the mutant MT1Δ*mmlL* indicates that the *mmlL* gene product probably is involved in the hydrolysis of the lactone ring and therefore that *mmlL* encodes a methylenol-lactone hydrolase, which is able to transform 4-methyl-3-oxoadipate enol-lactone into 4-methyl-3-oxoadipate. The accumulation of 3-ML rather than 4-methyl-3-oxoadipate-enol-lactone is explained by the reversibility of the MmlJ-catalyzed reaction, where the equilibrium favors the formation of the muconolactone (Fig. 3) (43).

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). From the sequence identity with functionally characterized 3-oxoadipate:succinly-CoA transferases (34, 44), it is reasonable to assume that the *mmlFG* gene products are re-

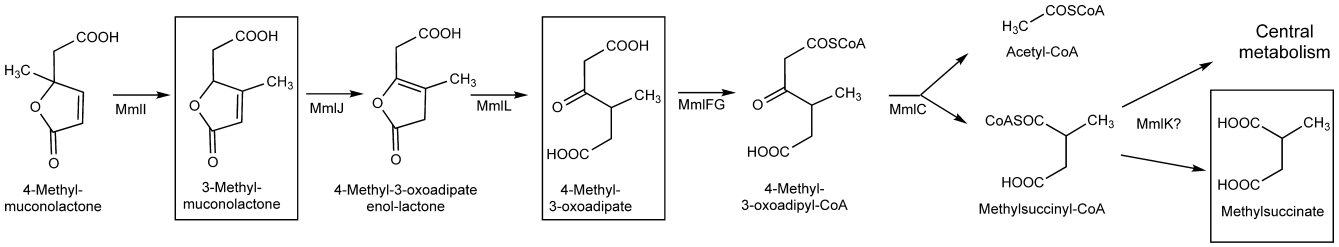


FIG. 3. Proposed pathway for 4-ML degradation by *P. reinekei* MT1. Metabolites identified in the current study are depicted in boxes. MmlI, 4-methylmuconolactone methylisomerase; MmlJ, methylmuconolactone isomerase; MmlL, 4-methyl-3-oxoadipate enol-lactone hydrolase; MmlFG, 4-methyl-3-oxoadipate-CoA transferase; MmlC, 4-methyl-3-oxoadipyl-CoA thiolase; MmlK, acetyl-CoA-transferase/hydrolase.

sponsible for transformation of 4-methyl-3-oxoadipate into 4-methyl-3-oxoadipyl-CoA (Fig. 3). Knockout mutants of *mmlF* and *mmlG* were not generated, since *pcaIJ* genes, which could eventually be recruited and thus mask the *mmlFG* mutant phenotype, are typically observed in *Pseudomonas* strains.

Subsequent transformation of 3-oxoadipyl-CoA via the 3-oxoadipate pathway is catalyzed by 3-oxoadipyl-CoA thiolase, forming succinyl-CoA and acetyl-CoA (Fig. 3). 3-Oxoadipyl-CoA thiolases have been biochemically characterized for various Gram-negative bacteria, including the 3-oxoadipyl-CoA thiolases of *P. knackmussii* B13 (34) or *P. putida* PRS2000 (44). Thus far, 3-oxoadipyl-CoA thiolases of Gram-positive organisms have not been characterized, although previous analysis of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP (26) and recent genome sequencing projects show the presence of orthologous genes located in protocatechuate catabolic gene clusters of rhodococci, such as *Rhodococcus jostii* RHA1, whose functionality has been supported by transcriptomic and proteomic analysis (45). The close phylogenetic relationship of MmlC with PcaF of rhodococci (see Fig. S5 in the supplemental material) and the accumulation of 4-methyl-3-oxoadipate by the mutant MT1Δ*mmlC* support the notion that this enzyme functions as a 4-methyl-3-oxoadipyl-CoA thiolase, transforming its substrate into methylsuccinyl-CoA and acetyl-CoA (Fig. 3). Whether 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 into the culture medium of the free acids rather than of CoA derivatives has been frequently reported (2) and has been suggested as a general strategy of bacterial cells to prevent the depletion of the intracellular CoA pool (42).

As indicated above, methylsuccinyl-CoA may be formed by MmlC during the degradation of 4-ML (Fig. 3). In fact, methylsuccinate is accumulated by the mutant MT1Δ*mmlK*, suggesting that methylsuccinate and/or its CoA derivative is a metabolite of 4-ML degradation. Information on the metabolic fate of methylsuccinate or methylsuccinyl-CoA is limited. Both compounds have been shown to occur 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 the case with bacteria, cycloisomerization of 3-methyl-*cis*,*cis*-muconate produces 3-ML directly, 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 for either genes or proteins involved in this process (51, 52). The 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 to be an intermediate in two pathways, the ethylmalonyl-CoA pathway for acetate assimilation in *Rhodobacter sphaeroides* (1, 25) and the glyoxylate regeneration cycle of *Methylobacterium extorquens* (36). In both cases, methylsuccinate is esterified at the C-1 carbon as an intermediate. In light of these observations, the metabolic fate of methylsuccinate in *P. reinekei* MT1 and whether *mmlK* encodes a methylsuccinyl-CoA hydrolase remain to be elucidated. A significant mechanistic difference between the 3-oxoadipate pathway and the 4-ML degradative pathway also has to be considered for future 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, it remains unclear whether methylsuccinyl-CoA is directly used by MmlC for thiolytic cleavage of 4-methyl-3-oxoadipy-CoA or whether succinyl-CoA is independently generated and MmlK encodes a CoA transferase involved in such reactions. Biochemical characterization of enzymes encoded by the *mml* cluster is currently being 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 the *mmlD* gene but also the *mmlC* and *mmlK* genes are absent from the *mml* clusters of *C. necator* JMP134 and H16. Since *C. necator* JMP134 has been reported to grow on 4-ML (47), the required genetic elements and their respective activities should be recruited from elsewhere on the genome. Even though 3-oxoadipyl-CoA thiolase from the 3-oxoadipate pathway is obviously 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 this enzyme is preceded by a gene (YP_840887) the putative gene product of which exhibits significant sequence identity (55%) with MmlK. The most closely related MmlC homologues in *C. necator* JMP134 are ReutA_1348 (YP_295562; 42% identity), which, based on its sequence identity and genomic context, can be assumed to be involved in polyhydroxyalkanoate formation, and ReutA_1355 (YP_295567; 43% identity). Whether 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 the *mmlC*, *mmlK*, and *mmlD* genes, their 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 archetype *mml* gene cluster was complemented by additional genes. Nevertheless, it should also be noted that proteins encoded by homologous genes share only 65 to 70% sequence identity. As an example, the level of identity between methylmuconolactone isomerases (65%) resembles those between muconolactone isomerases from *Pseudomonas* and *Cupriavidus* strains (54 to 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 from genome projects, an *mml* cluster with MmlI has been observed only in *P. reinekei* MT1, *C. necator* JMP134, and *C. necator*

H16. It should be stated, however, that currently available genomes give only a highly biased overview on bacterial metabolic properties. Taking into account the widespread distribution of the 3-oxoadipate pathway at least in the *Proteobacteria* plus the fact that catechol 1,2-dioxygenases and muconate cycloisomerases in general exhibit significant activity with methyl-substituted substrate analogues (8, 57, 60), it can be reasoned that in the environment, a significant amount of methyl-substituted aromatics are funneled into such a route and methylmuconolactone degraders could play an important role in further funneling these intermediates into the Krebs cycle.

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