Marín, M., Pérez-Pantoja, D., Donoso, R., Wray, V., González, B., Pieper, D.H.
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 mmlB. This reaction produces 3-ML and is followed by a rearrangement of the double bond catalyzed by the methylmuconolactone isomerase encoded by mmlI. 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 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 isomerasers 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 methylmuconolactone 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.
MT1 degrades 4-methylsalicylate via adipyl-CoA, although details of this reaction are not available. 3-ML-CoA has been proposed to proceed via 4-methyl-3-oxo-
this transformation are available. Further degradation of (12). Unfortunately, no gene or protein sequence data related
the synthesis of 3-ML-CoA from ATP, coenzyme A, and 3-ML
by a 3-methylmuconolactone-CoA synthetase, which catalyzes
ward methyl-substituted muconolactones has been observed as
(47). However, no typical enol-lactone hydrolase activity to-
may be transformed to 4-methyl-3-oxoadipate by a hydrolase
been proposed that in this strain, the enol-lactone intermediate
can 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-oxo-
adipyl-CoA, although details of this reaction are not available.
Recently Cámera et al. reported that Pseudomonas reinekei
MT1 degrades 4-methylsalicylate via ortho cleavage of 4-methyl-
catechol (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-substi-
tuted substrates, ensuring effective funneling of methyalizar-
atics into the ortho cleavage pathway. Additionally, P. reinekei
MT1 exhibits MmII activity (8), which indicates that methyl-
substituted aromatics are degraded via 4-ML. In contrast to C.
necator JMP134, which mineralizes methyalaromatics, such as
4-methyphenol, 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 metab-
olites 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-methyl-
benzoate 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
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. 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 (20
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). MmII activity was measured by
high-performance liquid chromatography (HPLC) following the transformation
of 4-ML to 3-ML as reported previously (50). Activity of MmII 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 locate the mmlJ gene cluster, part of the mmlJ gene was amplified by PCR using the degenerate primers NH3MMLF1 and NH3MMLR1, 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, NH3MMLF3, was designed and used in a second PCR round with a reverse degenerate primer, NH3MMLR4, designed from a sequence alignment of methylmuconolactones 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 NH3MMLF3 and NH3MMLR7, specific for the mmlJ gene. Positive clones were purified using the FosmidMAX DNA purification kit (Epipcent, Madison, WI) and subjected to direct sequencing. The upstream region of the mmlJ gene, using the ABI Prism BigDye Terminator v1.1 ready reaction cycle sequenc- ing 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 (5). Translated protein sequences were aligned with the BLASTX and CLC software programs. 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 A600 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 protease 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)] (Boehr- inger 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 electro- phoresis (CHEF) (15) using a CHEF-DRILL system (Bio-Rad, München, Ger- many). 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, fol- lowed 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 (A600 = 0.7), the culture was induced by addition of 0.5 mM 4-methylsalicylate and further incu- bated for 1 h. After addition of 8 ml RNAprotect reagent (Qiagen), total RNA was isolated by using the RNeasy minikit (Qiagen), according to the manufacturer’s instructions. The resulting RNA was quantified using a Picoquant fluorimeter (Ceratophyllum, 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/P1MT2, 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) deoxyribonucleoside triphosphates, 1 mM MgCl2, 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 (A600 = 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 P11J134/ 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 pKGWPO, a broad-range vector which was constructed as follows. The low background activity LacZ cassette and the multiple cloning site from plasmid pTZ120 (58) were amplified using the pTZ110LacZEFW 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) ac- cording to the manufacturer’s instructions. The integrity of the resulting plasmid was confirmed by restriction enzyme digestion and sequencing.

Putative promoter regions were fused to the lacZ reporter gene of pKGWPO as follows. A 258-bp PCR product comprising the bp 12 to 269 region upstream of the translational start site of the mmlJ gene of P. reinekei MT1 was amplified with the primers PmHydMT1FW and PmHydMT1RV. A 366-bp PCR product comprising the bp 12 to 377 region upstream of the translational start site of mmlL was amplified with the primers PmCAT_FW and PmCAT_RV. As a control, a 90-bp fragment was amplified using the primers pKGWP0, forming the plasmids pm_mmlJ and pm_mmlL, respectively. The lacZ fusion of the putative promoter region of the mmlJ 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 mmlJ. This region was amplified with the primers Pm11LFWc and Pm11LRV from the EcoRI/BamHI site of plasmid pS858. The resulting plasmid pS858 was digested with SfiI and cloned into pBGmml plasmid. The cassette was introduced into the chromosome of C. necator JMP134::X by biparental mating using E. coli S17/1 as a donor strain. Conjunctants 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 mmlN. gene deletion mutants were constructed with the previously described Flp-FLP recombi- nase target (FRT) recombinase 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-AccI, respectively) and cloned into the PstI-AccI restriction site of the pEX18Ap vector, forming the pABmml plasmid series. Subsequently, a 1.8-kb BamHI fragment from the p8858 plasmid carrying a Gm′-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 S17/1 as a donor strain. The transconjugants pABmml series were selected on ABC medium supplemented with 50 μg/ml Gm, and merodiploids were resolved by additional plating on ABC medium supplemented with 5% sucrose. Deletion of the Gm′-GFP cassette was achieved by conjugation of the Flp-expressing pBBFLP plasmid (19) into the resulting strains by biparental mating using E. coli CC118Sp (30) as a donor and selec-
tion on ABC medium containing Te. 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 MT1ΔmmlL mutant. The MT1ΔmmlL deletion mutant was separately complemented with the mml gene from P. reinekei MT1 and C. necator JMP134. The mml 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 plasmid pBBFLP, which was separately complemented with the plasmid pBBFLP and dissolved in 0.7 ml d6-acetone. Further samples for 1H NMR spectroscopy were prepared by addition of the metabolite to 1 ml of D6-H2O.

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 mml 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. 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.
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 mmlL and mmlI genes have a proven function in C. necator JMP134 (50, 53). The mmlL gene encodes MmlL, a unique enzyme belonging to the Mml protein family (PF09448). The predicted enzyme of P. reinekei MT1 shares 70% and 69% of sequence identity with MmlL of C. necator JMP134 and with the predicted MmlL protein of C. necator H16, the only homologues currently available from public databases. Phylogenetic analysis indicated that the mmlL gene product of P. reinekei MT1 is most closely related to the mmlL gene products of C. necator JMP134 and H16 but only distantly related to muconolate 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 transfersases 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 C6-C18 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 thiolysis 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 JMP134a

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>β-Galactosidase activity with inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>MT1(pm_mmlC)</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>MT1(pm_mmlLMT1)</td>
<td>310 ± 70</td>
</tr>
<tr>
<td>JMP134-X(pm_mmlLJMP134)</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>MT1ΔmmlL(pm_mmlC)</td>
<td>86 ± 23</td>
</tr>
<tr>
<td>MT1ΔmmlLJMP134(pm_mmlLMT1)</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>MT1ΔmmlCJMP134(pm_mmlC)</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>MT1ΔmmlCJMP134(pm_mmlLMT1)</td>
<td>290 ± 15</td>
</tr>
</tbody>
</table>

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 MT1Δmml transforms 4-ML quantitatively to 3-ML and P. reinekei MT1ΔmmlC transforms 4-ML quantitatively to 4-methyl-3-oxoadipate. Activities are expressed as Miller units.

The 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 both C. necator JMP134 and 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 oprfX-mmlC and mmlK-orpY 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-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 MT1ΔmmlL and P. reinekei MT1ΔmmlC, 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 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 MT1ΔmmlL, MT1ΔmmlC, and MT1ΔmmlK 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 MT1ΔmmlD 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 MT1ΔmmlL with plasmid pBS1mmlLMT1, harboring the mmlL gene of P. reinekei MT1, was performed in order to rule out possible polar effects. Furthermore, transcomplementation with pBS1mmlCJMP134, harboring the mmlC 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 MT1ΔmmlL, MT1ΔmmlC, and MT1ΔmmlK mutants, resting cell assays were performed using 1 mM 4-methylsalicylate as a substrate. HPLC and 1H NMR analysis revealed that the mutant MT1ΔmmlL transforms 4-methylsalicylate quantitatively into 3-ML, which accumulated after 24 h up to 1.13 ± 0.08 mM (Table 3). The mutants MT1ΔmmlC and MT1ΔmmlK transform 4-methylsalicylate without accumulation of UV-absorbing metabolites. Analysis by 1H NMR spectroscopy of cell-free supernatants after complete transformation of the substrate (6 h), as well as after extended incubation (24 h), indicated that MT1ΔmmlC accumulates a single metabolite, the 1H NMR spectrum of which was essentially identical to that previously described for the dimethylester of 4-methyl-3-oxoadipate (Table 3). 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 MT1ΔmmlK accumulates two metabolites. 1H 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 1H 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.
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, encoding these two enzymes are organized in a gene cluster, comprising 10 catabolic genes *mml* encoding 3-oxoadipate:succinyl-CoA transferases (34, 44), it is characterized 3-oxoadipate:succinyl-CoA transferases (termed PcaIJ) (43).

Previously it was proposed that the degradation of 3-ML in *C. necator* proceeds via a route analogous to the 3-oxoadipate pathway, where the equilibrium favors the formation of the muconolactone ring and therefore that the gene product probably is involved in the hydrolysis of 3-ML in the mutant MT1, 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 MT1 strains 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 PeaIJ or CatIJ). From the sequence identity with functionally characterized 3-oxoadipate:succinyl-CoA transferases (34, 44), it is reasonable to assume that the *mmlFG* gene products are re-
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 MT1ΔmmlC support the notion that this enzyme functions as a 4-methyl-3-oxoadipyl-CoA thiolase, transforming its substrate into methysuccinyl-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, methysuccinyl-CoA may be formed by MmlC during the degradation of 4-ML (Fig. 3). In fact, methysuccinate is accumulated by the mutant MT1ΔmmlK, suggesting that methysuccinate and/or its CoA derivative is a metabolite of 4-ML degradation. Information on the metabolic fate of methysuccinate or methysuccinyl-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 methysuccinate. Unfortunately, no sequence information is available for either genes or proteins involved in this process (51, 52). The metabolism of methysuccinyl-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. Methysuccinyl-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, methysuccinate is esterified at the C-1 carbon as an intermediate. In light of these observations, the metabolic fate of methysuccinate in P. reinekei MT1 and whether mmlK encodes a methysuccinyl-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 methysuccinyl-CoA is directly used by MmlC for thiolic cleavage of 4-methyl-3-oxoadipyl-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 poly-hydroxyalkanoate formation, and ReuTA_1355 (YP_295567; 43% identity). Whether these or other unrelated proteins carry out thiolic 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 methysuccinyl-CoA/methysuccinate 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 MmlII 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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