JOURNAL OF BACTERIOLOGY, Aug. 2009, p. 4905–4915 Vol. 191, No. 15
0021-9193/09/$08.00+0 doi:10.1128/JB.00331-09

Characterization of a Gene Cluster Involved in 4-Chlorocatechol Degradation by *Pseudomonas reinekei* MT1[▽]

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Received 10 March 2009/Accepted 17 May 2009

***Pseudomonas reinekei* MT1 has previously been reported to degrade 4- and 5-chlorosalicylate by a pathway with 4-chlorocatechol, 3-chloromuconate, 4-chloromuconolactone, and maleylacetate as intermediates, and a gene cluster channeling various salicylates into an intradiol cleavage route has been reported. We now report that during growth on 5-chlorosalicylate, besides a novel (chloro)catechol 1,2-dioxygenase, $C12O_{ccaA}$, a novel (chloro)muconate cycloisomerase, MCI_{ccaB}, which showed features not yet reported, was induced. This cycloisomerase, which was practically inactive with muconate, evolved for the turnover of 3-substituted muconates and transforms 3-chloromuconate into equal amounts of *cis*-dienelactone and protoanemonin, suggesting that it is a functional intermediate between chloromuconate cycloisomerases and muconate cycloisomerases. The corresponding genes, *ccaA* ($C12O_{ccaA}$) and *ccaB* (MCI_{ccaB}), were located in a 5.1-kb genomic region clustered with genes encoding *trans*-dienelactone hydrolase (*ccaC*) and maleylacetate reductase (*ccaD*) and a putative regulatory gene, *ccaR*, homologous to regulators of the IclR-type family. Thus, this region includes genes sufficient to enable MT1 to transform 4-chlorocatechol to 3-oxoadipate. Phylogenetic analysis showed that $C12O_{ccaA}$ and MCI_{ccaB} are only distantly related to previously described catechol 1,2-dioxygenases and muconate cycloisomerases. Kinetic analysis indicated that MCI_{ccaB} and the previously identified $C12O_{salD}$, rather than $C12O_{ccaA}$, are crucial for 5-chlorosalicylate degradation. Thus, MT1 uses enzymes encoded by a completely novel gene cluster for degradation of chlorosalicylates, which, together with a gene cluster encoding enzymes for channeling salicylates into the *ortho*-cleavage pathway, form an effective pathway for 4- and 5-chlorosalicylate mineralization.**

The aerobic degradation of chloroaromatic compounds usually proceeds via chlorocatechols as central intermediates (20, 47), which in most of the cases reported thus far, are further degraded by enzymes of the chlorocatechol pathway (44). This pathway involves *ortho*-cleavage by a chlorocatechol 1,2-dioxygenase with high activity for chlorocatechols (12), a chloromuconate cycloisomerase with high activity for chloromuconates (54), a dienelactone hydrolase active with both *cis*- and *trans*-dienelactone (4-carboxymethylenebut-2-en-4-olide) (54), and a maleylacetate reductase (MAR) (28).

However, it has become evident in recent years that microorganisms have evolved various alternative strategies to mineralize chlorocatechols. *Pseudomonas putida* GJ31 was found to degrade chlorobenzene rapidly via 3-chlorocatechol using a catechol *meta*-cleavage pathway (33). Two alternative pathways for 3- and 4-chlorocatechol degradation that involve reactions known from the chlorocatechol, as well as the 3-oxoadipate, pathway have recently been observed in *Rhodococcus opacus* 1CP (35) and *Pseudomonas reinekei* MT1 (39). In *R. opacus* 1CP, 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate (the ring cleavage products of 4-chlorocatechol and 3,5-dichlorocatechol, respectively) are converted to the respective *cis*-dienelactones (35, 58), similar to the reaction described for proteobacterial chloromuconate cycloisomerases (54). However, proteobacterial chloromuconate cycloisomerase can dehalogenate 2-chloromuconate (the ring cleavage product of 3-chlorocatechol) and transform this compound via 5-chloromuconolactone into *trans*-dienelactone (54, 65), whereas none of the described chloromuconate cycloisomerases of *R. opacus* 1CP can catalyze such a dehalogenation, and 5-chloromuconolactone is the product of the cycloisomerization reaction (35, 58). Dehalogenation is achieved by an enzyme with high sequence similarity to muconolactone isomerases (35), which in proteobacteria have been shown to be capable of dehalogenating 5-chloromuconolactone to *cis*-dienelactone (46).

In *P. reinekei* MT1, a *trans*-dienelactone hydrolase (*trans*-DLH) was identified as the key enzyme involved in the degradation of 4- and 5-chlorosalicylate via 4-chlorocatechol as an intermediate (39). In contrast to all previously described dienelactone hydrolases involved in chlorocatechol degradation, which belong to the α/β hydrolase fold enzymes with a catalytic triad consisting of Cys, His, and Asp (10), *trans*-DLH was shown to be a zinc-dependent hydrolase (8). The function

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▽ Published ahead of print on 22 May 2009.

of this enzyme in the 4-chlorocatechol metabolic pathway was to interact with the muconate cycloisomerase (MCI)-mediated transformation of 3-chloromuconate into protoanemonin. By acting on the reaction intermediate 4-chloromuconolactone, *trans*-DLH prevents the formation of protoanemonin by catalyzing its hydrolysis to maleylacetate (39). Maleylacetate, in turn, is reduced by MAR to 3-oxoadipate.

A more detailed genetic and biochemical analysis of the degradation of differently substituted salicylates (7) had shown the presence of two catabolic gene clusters in MT1. An archetype *catRBCA* gene cluster was shown to be involved in salicylate degradation. The second gene cluster (*sal*) had a novel gene arrangement, with *salA*, encoding a salicylate 1-hydroxylase, clustered with the *salCD* genes, encoding MCI and catechol 1,2-dioxygenase (C12O), respectively. As these genes were expressed during growth on differently substituted salicylates, it was proposed that the function of the *sal* gene cluster is to channel both chlorosubstituted and methylsubstituted salicylates into a catechol *ortho*-cleavage pathway, followed by dismantling of the formed substituted muconolactones through specific pathways. However, previous analyses had indicated the presence of an additional and thus third (chloro)muconate cycloisomerase in MT1 during growth on chlorosalicylate, which is distinct from both previously described MCIs encoded by the *cat* cluster (MCI_{catB}) and the *sal* cluster (MCI_{salC}), as it transforms 3-chloromuconate into approximately equal amounts of *cis*-dienelactone and protoanemonin (39). In the present report, this cycloisomerase is biochemically and genetically described and shown to be located in a third gene cluster involved in the degradation of 5-chlorosalicylate by strain MT1. This cluster comprises genes encoding a third C12O, *trans*-DLH (8), and a MAR. Evidently, *P. reinekei* MT1 is the first microorganism in which such a complex net of genes involved in chlorocatechol degradation has been described.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. reinekei* MT1 was grown and cell extracts were prepared as previously described (39).

Enzyme assays. C12O, MCI, *trans*-DLH, and MAR activities were determined spectrophotometrically as previously described (27, 39, 54). The activity of MCI_{ccaB} with 3-chloromuconate was determined by high-performance liquid chromatography (HPLC) (39) following substrate depletion and product formation. To more sensitively follow the activity of MCI_{ccaB} with muconate and 2-chloromuconate, the transformation of these substrates (100 μM) was also followed by HPLC using up to 10 U/ml (measured with 100 μM 3-chloromuconate) of purified MCI_{ccaB}. Specific activities are expressed as μmol of substrate converted or product formed per minute per gram of protein at 25°C. Protein concentrations were determined by the Bradford procedure using the Bio-Rad protein assay with bovine serum albumin as a protein standard (5).

Analysis of kinetic data. The V_{max}, k_{cat}, and apparent K_m values of $C12O_{ccaA}$ with catechol, 3-methylcatechol, 4-methylcatechol, and 4-chlorocatechol were determined using 1 to 100 μM of substrate in air-saturated buffer, and the kinetic data were calculated from the initial velocities using the Michaelis-Menten equation by nonlinear regression (KaleidaGraph; Synergy Software). As very low K_m values were indicated by this method, kinetic data were finally determined from progress curves obtained from reactions with initial substrate concentrations of 10 μM, as previously described (7). V_{max}, k_{cat}, and apparent K_m values of MCI_{ccaB} with 2-methylmuconate, and 3-methylmuconate were determined using 2 to 100 μM of substrate. Transformation of 3-chloromuconate was determined by HPLC analysis at substrate concentrations of 50 μM to 500 μM. Samples were taken during the reaction time, and the formation of protoanemonin and *cis*-dienelactone was directly quantified by HPLC analysis. At least two independent experiments were performed for each value. K_m and V_{max} values were calculated by nonlinear regression to the Michaelis-Menten equation, using KaleidaGraph (Synergy Software). Turnover numbers (k_{cat} values) were calculated assuming subunit molecular masses of 29,424 ($C12O_{ccaA}$) and 39,764 (MCI_{ccaB}) Da, respectively.

Enzyme purification. $C12O_{ccaA}$ and MCI_{ccaB} were purified using a Fast Protein Liquid Chromatography system (Amersham Biosciences). Cells were harvested during late exponential growth with 5-chlorosalicylate or 4-methylsalicylate. Cell disruption and all protein elutions were performed in 50 mM Tris-HCl, pH 7.5, 2 mM $MnCl_2$.

For analyzing the presence and abundances of different C12Os and MCIs under different growth conditions, either cell extracts (usually containing 35 mg of protein per ml) were applied directly to a MonoQ HR5/5 (Amersham Pharmacia Biotech) and proteins were eluted by a linear gradient of 0 to 0.5 M NaCl over 25 ml with a flow of 0.5 ml/min, or the cell extract was mixed with 4 M $(NH_4)_2SO_4$ to give a final concentration of 1 M $(NH_4)_2SO_4$ and applied to a Source 15PHE PE 4.6/100 (hydrophobic interaction) column (Amersham Pharmacia Biotech). Proteins were eluted from the Source column by a linear gradient of $(NH_4)_2SO_4$ (1 M to 0 M) over 25 ml with a flow of 0.5 ml/min. Fraction volumes were 0.5 ml. Hydrophobic interaction chromatography (HIC) separated $C12O_{ccaA}$ (0.52 ± 0.02 M), $C12O_{salD}$ (0.45 ± 0.04 M), $C12O_{catA}$ (0.16 ± 0.04 M), MCI_{ccaB} (0.25 ± 0.04 M), MCI_{salC} (0.06 ± 0.06 M), and MCI_{catB} (0.12 ± 0.06 M), thus excluding interference between their activities. During anion-exchange chromatography, $C12O_{ccaA}$ eluted at 0.23 ± 0.01 M NaCl, whereas MCI_{ccaB} eluted at 0.37 ± 0.02 M NaCl. Under these conditions, $C12O_{catA}$ and $C12O_{salD}$ had been shown to coelute at 0.28 ± 0.02 M NaCl, whereas MCI_{catB} and MCI_{salC} coeluted at 0.24 ± 0.02 M NaCl (7).

For purification of $C12O_{ccaA}$, 35 mg of protein from 5-chlorosalicylate-grown cells was applied to the MonoQ HR 5/5 (Amersham Pharmacia Biotech), and proteins were eluted as described above. Fractions containing $C12O_{ccaA}$ activity were combined, supplemented with 4 M $(NH_4)_2SO_4$ to give a final concentration of 1 M $(NH_4)_2SO_4$, and loaded on a Source 15PHE PE 4.6/100 (hydrophobic interaction) column (Amersham Pharmacia Biotech) as described above.

For purification of MCI_{ccaB}, up to 400 mg of protein from 5-chlorosalicylate-grown cells was applied to a MonoQ HR 10/10 (Amersham Pharmacia Biotech). A stepwise gradient of 0 to 60 mM NaCl over 40 ml, 60 to 380 mM NaCl over 120 ml, and 380 to 2,000 mM NaCl over 40 ml was applied. The flow rate was 0.3 ml/min. The eluate was collected in fractions of 5 ml. All fractions eluting at NaCl concentrations of 90 to 330 mM were pooled and concentrated to a final volume of 4.25 ml using ultrafiltration by Centriprep YM-50 (Millipore) according to the protocol of the manufacturer. The protein solution was supplemented with 4 M $(NH_4)_2SO_4$ to give a final concentration of 0.8 M $(NH_4)_2SO_4$ and centrifuged directly before application of the soluble proteins to the Source column. Aliquots comprising 40 mg of protein were separated as described above. Fractions containing MCI_{ccaB} were combined and concentrated by a Centricon YM-50 (Millipore). Further purification was achieved by gel filtration using a Superose 12 HR10/10 column (Amersham Pharmacia Biotech). Proteins were eluted with 50 mM Tris-HCl, 2 mM $MnCl_2$, pH 7.5, over 15 ml (flow rate, 0.2 ml/min; fraction volume, 0.5 ml). The fractions containing high MCI_{ccaB} activity (eluting at 10.5 to 11.5 ml) were applied to a MonoQ HR5/5 (anionic-exchange) column (Amersham Pharmacia Biotech), and the proteins were eluted by a linear gradient of 0 to 0.4 M NaCl over 25 ml with a flow of 0.2 ml/min. Homogeneity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *trans*-DLH was purified as previously described (8).

Transformation of 3-chloromuconate by enzyme mixtures. Product formation from 3-chloromuconate by purified MCI_{ccaB} and in the presence of purified *trans*-DLH was analyzed by HPLC in assays performed at room temperature in 150 μl Tris-HCl (50 mM), 2 mM $MnCl_2$, pH 7.5, with 120 μM 3-chloromuconate as a substrate. MCI_{ccaB} was added to give an activity of 53 mU/ml (determined by the transformation of 100 μM 3-chloro-*cis*,*cis*-muconate), corresponding to 8.8 nM MCI_{ccaB}, whereas *trans*-DLH was applied in amounts ranging from 1.32 to 1,320 mU/ml (determined by the transformation of 50 μM *trans*-dienelactone), corresponding to 0.88 to 88 nM *trans*-DLH.

Determination of molecular mass. The molecular mass of MCI_{ccaB} was determined by gel filtration using a Superose 12 column as described above. The column was calibrated for molecular mass determinations using ovalbumin (43 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa) from Bio-Rad.

Electrophoretic methods. SDS-PAGE was performed on a Bio-Rad Miniprotein II as previously described (32), with acrylamide concentrations of 5 and 10% (wt/vol) used for the concentrating and separating gels, respectively. The proteins were stained with Coomassie brilliant blue (Serva). A PageRuler Protein Ladder (Fermentas) was used as a marker.

Amino acid sequencing. N-terminal amino acid sequences were determined as described previously (26).

Identification of the gene encoding MCI_{ccaB} of strain MT1. Part of the gene encoding MCI_{ccaB} was amplified by PCR using the degenerate primers NT1B (WSNCARGGNTTYGTNATCGG) and NTREV2A (AANWSCATNCKDAT NGGCTG), which were designed based on the determined N-terminal protein sequence (underlined) SQGFVIGRVLAQRLDIPFSQPIRMSFGTLD. Touchdown PCR consisted of an initial denaturation (94°C for 4 min), followed by 10 cycles of denaturation (94°C for 45 s), annealing (60°C for 30 s − 1°C per cycle), and elongation (72°C for 30 s), followed by 25 cycles with an annealing temperature of 50°C for 45 s and a final elongation step (72°C for 7 min). A 72-bp fragment was obtained, cloned into the pGEM-T Easy vector (Promega), and transformed into *Escherichia coli* XL10-Gold (Stratagene), and inserts of the clones generated were then sequenced. The deduced amino acid sequence matched that of the N-terminal amino acid sequence.

An extended part of the gene encoding MCI_{ccaB} was amplified by PCR using the primers MCIB1 (GCAACGGCTGGATATACCTT) and MCIBR2 (GTRT CGCCRCTSGCSARCGTCC), which were designed based on the DNA sequence generated above and a protein sequence, WTLASGDT, identified by protein sequence alignment to be conserved in proteobacterial muconate and chloromuconate cycloisomerases. The touchdown PCR conditions included 10 cycles as described above, followed by 25 cycles at an annealing temperature of 55°C. An approximately 400-bp fragment was obtained, cloned, and sequenced as mentioned above. The DNA sequence matched the sequence deduced from the N-terminal sequence, clearly confirming that the cloned PCR product corresponded to part of the gene encoding MCI_{ccaB}.

DNA isolation, fosmid library construction, and identification of the *cca* gene cluster. Preparation of the fosmid library in pCC1FOS, which comprised a total of 282 individual clones, was previously described (7). The fosmid library was screened by PCR using primers specific for *ccaB* (MCIB1 [GCAACGGCTGG ATATACCTT] and inMCIB [AGCAGAAACACCCAACTGCT], with an annealing temperature of 59°C). Fosmid clones harboring the expected 340-bp *ccaB* gene fragment were subsequently checked by PCR for the presence of the *ccaC* gene, encoding *trans*-DLH (TransFOR [AATCCCTGCCGACATACA AG] and TransREV [CGTCAGCATGAAGGTGTAGC]). From the three fosmids carrying both the *ccaB* and the *ccaC* gene fragments, one fosmid was chosen and purified with the FosmidMAX DNA purification kit (Epicentre), and the complete *cca* gene cluster was obtained by direct sequencing (Seqlab, Göttingen, Germany) from the purified fosmid with a sixfold coverage of the insert.

DNA sequencing and sequence analysis. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced using the ABI Prism BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and a DNA capillary sequencer, the 3130xl Genetic Analyzer (Applied Biosystems). Raw sequence data from both strands were assembled with Sequencher software version 4.0.5 (Gene Codes Corporation). DNA and protein similarity searches were performed using the BLASTN and BLASTP programs from the NCBI website. Translated protein sequences were aligned with CLUSTALX 1.83 using default values (61). The evolutionary history was inferred with MEGA4 (59) using the neighbor-joining algorithm 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.

Gene expression studies. Harvest of *P. reinekei* MT1 cells and RNA extraction were done as previously described (7). Reverse transcription (RT) and quantitative real-time PCR were performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) for one-step RT-PCR in a Rotor-Gene 2000 real-time PCR machine (Corbett Research). Transcripts of *ccaA*, *ccaB*, *ccaC*, and *ccaD* were quantified with the following primer pairs: CcaA-F (GGGCGCTTTCACACCA ATGACC) and CcaA-R (GCAGGTGAGCGGGTCGGAAGTA), CcaB-F (GC AGTTGAGGCGGCGGTTGTTA) and CcaB-R (GCTTGCCAACCAGGTCG AATGC), CcaC-F (TGACACGTCCAAATCCCTGCCG) and CcaC-R (GCAA GCGTGCGGCGTTATCAAT), and CcaD-F (GATGGCGTTGTCGGTCT TGG) and CcaD-R (TGACGGTTTCAGGGCGGATA). A housekeeping reference gene (ribosomal *rpsL*) was selected to normalize the results obtained (9, 13). Real-time PCRs were carried out and relative expression ratios were determined as previously described (7).

Mathematical calculations. Numerical calculations were performed with a kinetic model built in SIMULINK v6.4.1 under the MATLAB v7.2.0.232 environment (The MathWorks, Inc., Natick, MA) based on Michaelis-Menten kinetics using the kinetic constants experimentally determined here or previously (7) and assuming a constant concentration of enzyme and zero-order kinetics for oxygen and NADH.

Analytical methods. HPLC was performed as previously described (7).

Chemicals. 3-Chlorocatechol, 4-chlorocatechol, 3-methylcatechol, and 4-methylcatechol were obtained from Helix Biotech. 2-Methylmuconate, 3-methylmuconate, and 3-chloro-*cis*,*cis*-muconate were freshly prepared from 3-methylcatechol, 4-methylcatechol, and 4-chlorocatechol, respectively, in 50 mM Tris-HCl, pH 7.5, 2 mM $MnCl_2$ using chlorocatechol 1,2-dioxygenase TetC of *Pseudomonas chlororaphis* RW71 (45) or partially purified $C12O_{salC}$ free of muconate cycloisomerizing activity. *cis*-Dienelactone was kindly provided by Walter Reineke (Bergische Universität-Gesamthochschule, Wuppertal, Germany) and Stefan Kaschabeck (TU Bergakademie, Freiberg, Germany). Protoanemonin, 2-chloro-*cis*,*cis*-muconate, and *trans*-dienelactone were prepared as previously described (4, 48).

Nucleotide sequence accession number. The nucleotide sequence reported in this study was deposited in the DDBJ/EMBL/GenBank databases under accession number EF159980.

RESULTS

Characterization of a cycloisomerase transforming 3-chloromuconate into both *cis*-dienelactone and protoanemonin. Two MCIs, both transforming 3-chloromuconate into protoanemonin, with minor quantities of *cis*-dienelactone, had previously been characterized from *P. reinekei* MT1, and the encoding genes had been localized (7). However, during growth on 5-chlorosalicylate, the presence of a distinct enzyme capable of transforming 3-chloromuconate was evident. This enzyme, termed MCI_{ccaB}, eluted at 0.25 ± 0.04 M during HIC, and as previously indicated (39), approximately equal amounts of protoanemonin (50% ± 3%) and *cis*-dienelactone (47% ± 5%) were formed when proteins of such fractions were supplemented with 3-chloromuconate. As the formation of such a product mixture by any muconate or chloromuconate cycloisomerase had not been previously observed, the enzyme was purified to homogeneity. The native molecular mass of MCI_{ccaB} was estimated by gel filtration to be 350 ± 20 kDa, and a single band of 43 ± 3 kDa was observed on SDS gels. Thus, MCI_{ccaB}, like MCI of *P. putida* PRS2000 (22) or chloromuconate cycloisomerase from *Cupriavidus necator* JMP 134 (23), may be a homo-octamer. N-terminal amino acid analysis (SQGFVIGRVLAQRLDIPFSQPIRMSFGTLD) revealed no significant similarity when these sequences were compared to the sequences of other cycloisomerases available in databases.

Of the substrates tested, only 3-chloromuconate and 3-methylmuconate were transformed with high activity by this enzyme. The highest turnover rate, 10-fold higher than with 3-methylmuconate, was observed with 3-chloromuconate (Table 1). However, the specificity constants of 3-chloromuconate and 3-methylmuconate were almost equal, due to the significantly higher K_m value with 3-chloromuconate. Activity of the enzyme with muconate was negligible, and at a substrate concentration of 0.1 mM substrate, the activity was only 0.4% of that with 3-chloromuconate. Thus, from the substrate utilization profile, MCI_{ccaB} is clearly different from previously reported MCIs, which are characterized by their high activity with muconate (53, 54). It also differed from MCI_{salC} of MT1, which has previously been characterized as being adapted for the turnover of 3-methylmuconate (7) but retained a significant activity with muconate. MCI_{ccaB} was practically inactive with 2-chloromuconate, which is transformed at high rates by most proteobacterial chloromuconate cycloisomerases described thus far (31, 63, 64).

The fact that purified MCI_{ccaB} transformed 3-chloromuconate stoichiometrically into equal amounts of protoanemonin and *cis*-dienelactone contrasts with all previously described cycloisomerases, which form either protoanemonin (MCIs) or *cis*-dienelactone (chloromuconate cycloisomerases) as the predominant product (4, 39, 53, 54, 58). Following 3-chloromu-

TABLE 1. Substrate specificities of $C12O_{ccaA}$ and MCI_{ccaB} from *P. reinekei* MT1[a]

Enzyme	Substrate	Activity with 0.1 mM substrate (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
$C12O_{ccaA}$	Catechol	2.5 ± 0.1	2.0 ± 0.4	1.2 ± 0.05	0.6
	4-Chlorocatechol	0.24 ± 0.02	0.6 ± 0.1	0.12 ± 0.01	0.2
	3-Chlorocatechol	0.08 ± 0.01	ND	ND	ND
	4-Methylcatechol	24.0 ± 1.2	0.6 ± 0.1	11.5 ± 0.6	19.2
	3-Methylcatechol	12.4 ± 0.3	21.5 ± 2.5	6.0 ± 0.15	0.3
MCI_{ccaB}	Muconate	0.55 ± 0.1	ND	ND	ND
	3-Chloromuconate	140 ± 10	105 ± 15	111 ± 8	1.1
	2-Chloromuconate	<0.003	ND	ND	ND
	3-Methylmuconate	26 ± 2.2	10.6 ± 1.2	11 ± 0.5	1.0
	2-Methylmuconate	0.95 ± 0.1	40 ± 8	0.5 ± 0.05	0.01

[a] The kinetic constants were determined as described in Materials and Methods. Standard deviations were calculated with the KaleidaGraph program. ND, not determined.

conate transformation over time showed that both products were formed at a constant ratio, indicating that the reaction mechanism was independent of the substrate concentration.

It has previously been shown that *trans*-DLH of strain MT1 interferes with the cycloisomerization of 3-chloromuconate catalyzed by MCI_{salC} (39), an enzyme encoded by the *sal* cluster and induced during growth on chlorosalicylates (7), and it was suggested that *trans*-DLH acts on intermediate 4-chloromuconolactone to form maleylacetate, thereby preventing protoanemonin formation. To validate the notion that *trans*-DLH can similarly interact with MCI_{ccaB}, 3-chloromuconate (0.12 mM) was transformed by enzyme mixtures comprising MCI_{ccaB} (8.8 nM) and various amounts of *trans*-DLH (0.88 to 88 nM). As previously observed for MCI_{salC} (39), the simultaneous presence of *trans*-DLH decreased the amount of protoanemonin formed (Fig. 1) but did not influence the extent of *cis*-dienelactone formation, which was always 47% ± 5% of the substrate transformed.

Characterization of a C12O specifically induced during growth on 5-chlorosalicylate. As an MCI that was not encoded by the previously described *cat* or *sal* gene cluster was induced during growth on chlorosalicylate (7), we assessed whether a distinct C12O was also induced under such conditions. In fact, C12O activity was observed in protein fractions of cell extracts, eluting at 0.23 ± 0.01 M NaCl during anionic-exchange chromatography, in addition to previously described $C12O_{salC}$, eluting at 0.29 ± 0.01 M NaCl. HIC confirmed the presence of a previously uncharacterized catechol dioxygenase, termed $C12O_{ccaA}$, eluting at 0.52 ± 0.02 M $(NH_4)_2SO_4$ in 5-chlorosalicylate-grown cells.

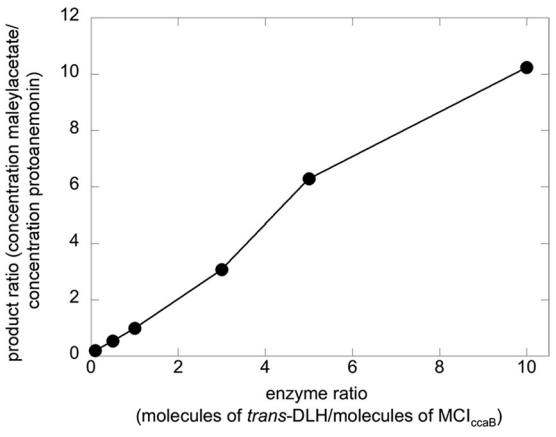


FIG. 1. Ratio of maleylacetate and protoanemonin formed from 3-chloromuconate by mixtures of MCI_{ccaB} (8.8 nM) with various amounts of *trans*-DLH (0 to 88 nM) of *P. reinekei* MT1. The reaction mixtures contained 50 mM Tris-HCl, 2 mM $MnCl_2$, pH 7.5, and 120 μM 3-chloromuconate. Substrate and product concentrations were analyzed by HPLC.

$C12O_{ccaA}$ was purified to 95% purity by a two-step procedure (see Materials and Methods). A prominent band of 30 ± 2 kDa observed after SDS-PAGE was subjected to N-terminal sequencing. The determined N terminus (AVSRLAELVTAL ESD) showed no significant similarity to any proteins in public databases. It thus seems that $C12O_{ccaA}$ is only distantly related to previously characterized C12Os.

Kinetic data were measured directly in fractions comprising $C12O_{ccaA}$ with a purity of at least 95% of the total protein. Thus, it can be calculated that maximum turnover rates with catechol of 2,375 U/g of protein correspond to activities of 2,500 ± 100 U/g $C12O_{ccaA}$ and, based on a subunit molecular mass of 29.424 kDa (as supposed for the predicted amino acid sequence of $C12O_{ccaA}$ [see below]), to a k_{cat} value for catechol of 1.2 ± 0.05 s^{-1} (Table 1). This was approximately 1 order of magnitude lower than those previously reported for $C12O_{catA}$ and $C12O_{salD}$ and for other previously analyzed proteobacterial C12Os (6, 37, 49, 51). A high turnover rate was observed only for 4-methylcatechol, and a comparison of specificity constants (k_{cat}/K_m) showed 4-methylcatechol to be the highly preferred substrate (Table 1). A similar substrate profile has so far been observed only for $C12O_{salD}$, and it contrasts with that reported for either catechol or chlorocatechol 1,2-dioxygenases (3, 6, 11, 45). However, the degree of specificity of $C12O_{ccaA}$ was even more remarkable than that of $C12O_{salD}$, as specificity constants for 4-methylcatechol compared to those for catechol, 4-chlorocatechol, and 3-methylcatechol differed by factors of 30 to 100. Surprisingly, activity of $C12O_{ccaA}$ against 4-chlorocatechol was rather poor and was similar to those of previously described C12Os (11, 30, 38, 51).

Characterization of the *cca* gene cluster. To localize genes encoding $C12O_{ccaA}$ and MCI_{ccaB}, degenerate primers based on the N-terminal sequence were used for the amplification from genomic DNA of a 72-bp DNA segment encoding part of

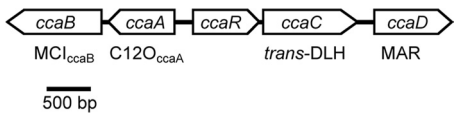


FIG. 2. Gene organization of a 5,129-bp region from *P. reinekei* MT1 containing the *cca* gene cluster. The arrows indicate gene orientations: *ccaA*, C12O gene; *ccaB*, MCI gene; *ccaC*, *trans*-DLH gene; *ccaD*, putative MAR gene; and *ccaR*, putative transcriptional regulator gene. The encoded enzymes are given below the gene clusters.

MCI_{ccaB}. This allowed the design of a specific primer that, together with a degenerate primer based on a conserved sequence motif identified in both proteobacterial muconate and chloromuconate cycloisomerases, resulted in the amplification of an ~400-bp DNA fragment. PCR-based screening of a fosmid library of the genome of strain MT1 using primers specific for the gene encoding MCI_{ccaB} and that encoding *trans*-DLH (8) showed that both genes were carried on the same fosmid, which contained an approximately 37.6-kb DNA fragment from MT1.

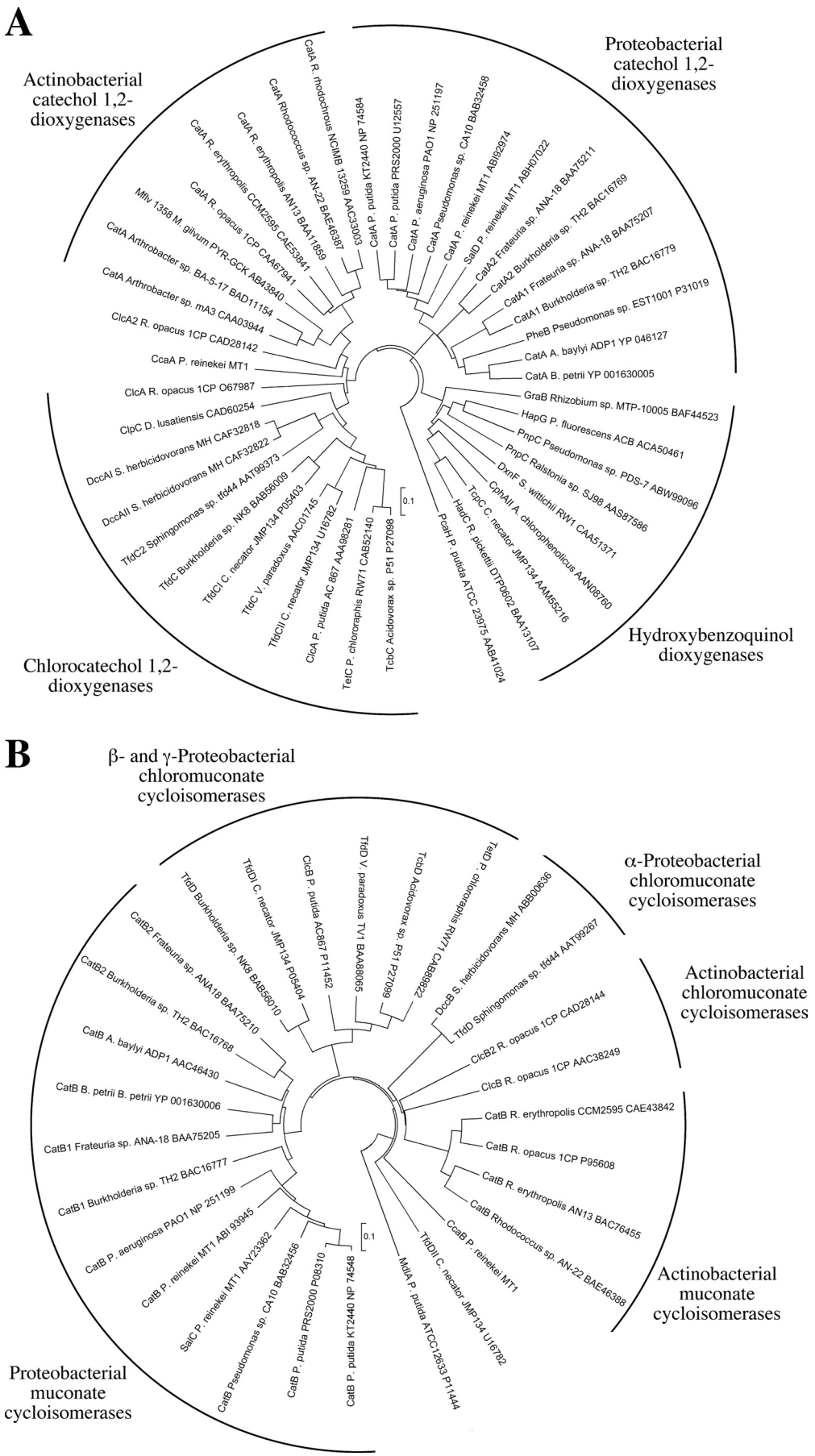
Sequencing of the insert revealed an approximately 5,100-bp region with five open reading frames (ORFs) (Fig. 2) probably involved in the degradation of aromatic compounds by strain MT1. One ORF, designated *ccaB*, contained the above-identified 400-bp fragment encoding part of MCI_{ccaB} and can thus be supposed to encode MCI_{ccaB}. The *ccaB* gene product showed only moderate identity to proteobacterial MCIs (35% to 42%), proteobacterial chloromuconate cycloisomerases (33% to 40%), or muconate and chloromuconate cycloisomerases (35% to 37%) identified in gram-positive microorganisms, which in a phylogenetic analysis form separate branches with low sequence identity to one another (Fig. 3). This indicated that MCI_{ccaB} of strain MT1 forms a new branch, illustrating a distinct evolutionary history. Upstream of *ccaB*, *ccaA* encoded an enzyme with a deduced N-terminal sequence identical to that of the above-characterized $C12O_{ccaA}$ protein. As observed for MCI_{ccaB}, in a phylogenetic analysis, $C12O_{ccaA}$ does not cluster with any of the previously described separate branches observed in intradiol dioxygenases (Fig. 3) and showed only moderate identities with proteobacterial C12Os (30% to 38%), proteobacterial chlorocatechol 1,2-dioxygenases (32% to 37%), or catechol and chlorocatechol 1,2-dioxygenases (31% to 43%) from gram-positive microorganisms. Lower sequence identity (27% to 33%) was observed with members of the hydroxyquinol branch of intradiol dioxygenases (1, 17). The predicted amino acid sequence of the ORF transcribed divergently toward *ccaA* and designated *ccaR* showed up to 47% sequence identity with identified and putative transcriptional regulators of the IclR family, specifically with those of the PobR subfamily of IclR-type regulators, comprising, among others, proteins involved in the transcriptional regulation of protocatechuate or 4-hydroxybenzoate degradative genes (62). The highest sequence identity was observed with a putative IclR regulator of *Corynebacterium efficiens* YS-314 (accession number BAC19104); however, only slightly lower sequence identity was observed with regulators with identified functions (40% sequence identity with *pcaR* of *P. putida* PRS2000, involved in regulation of protocatechuate degradation [50], and 39% sequence identity with *pcaR* of *P. putida* WCS358 [2]).

Downstream of *ccaR*, the previously described gene encoding *trans*-DLH (8) and designated *ccaC* could be localized. The deduced product of the downstream *ccaD* gene showed the highest sequence homology with MARs, with the highest identity (59%) being observed with MAR TfdF2 of the 2,4-dichlorophenoxyacetic acid-degrading *Sphingomonas* sp. strain TFD44 (60).

RT-PCR analysis of the *cca* cluster. The accumulation of transcripts of *ccaA*, *ccaB*, *ccaC*, and *ccaD* was measured during growth on 5-chlorosalicylate, salicylate, and acetate (noninducing negative control). When the relative expression levels between the target and the reference gene (*rpsL*) were compared to those under noninducing conditions (at a ratio of 1), significantly higher levels of *ccaA*, *ccaB*, *ccaC*, and *ccaD* transcripts were observed only in 5-chlorosalicylate-grown cells (50- to 150-fold) and not in salicylate-grown cells (Fig. 4).

Induction of $C12O_{ccaA}$ and MCI_{ccaB} during growth on 5-chlorosalicylate and 4-methylsalicylate. As two of the three C12O-encoding catabolic gene clusters of strain MT1 (the *sal* gene cluster and the *cca* gene cluster) were expressed during growth on 5-chlorosalicylate, the importance of the encoded C12Os and MCIs was assessed after growth on 5-chlorosalicylate and 4-methylsalicylate. Cell extracts were separated by anionic-exchange chromatography, fractions were monitored for transformation of 4-methylcatechol and 3-methylmuconate, and the activities were quantified. Both C12O and muconate cycloisomerizing activities could be nearly quantitatively recovered (recovery was >90% for C12O activity against 4-methylcatechol and 85 to 95% for MCI activity against 3-methylmuconate).

Fractions of cell extracts of 5-chlorosalicylate-grown cells eluting at 0.23 ± 0.01 M NaCl and thus containing $C12O_{ccaA}$ accounted for only 20% ± 5% of the total activity against 0.1 mM 4-methylcatechol, whereas fractions eluting at 0.28 ± 0.02 M NaCl and corresponding to $C12O_{salD}$ accounted for 80% ± 5% of the total activity against 0.1 mM 4-methylcatechol (Fig. 5). Analysis of cell extracts from 4-methylsalicylate-grown cells showed that only 7% ± 2% of the total activity against 4-methylcatechol was due to $C12O_{ccaA}$. Similar results were obtained when activities against 0.1 mM 3-methylmuconate were analyzed, with only 7% ± 2% (cell extracts of 5-chlorosalicylate-grown cells) and 4% ± 1% (cell extracts of 4-methylsalicylate-grown cells) of the total activity due to MCI_{ccaB}. This indicated that $C12O_{ccaA}$ and MCI_{ccaB} were of only minor importance during the degradation of 4-methylsalicylate. In contrast, a calculation of the respective activities against 0.1 mM 3-chloromuconate indicated that 75% ± 5% of the total activity in extracts of 5-chlorosalicylate-grown cells was due to induction of MCI_{ccaB}, whereas $C12O_{ccaA}$ seemed to be of minor importance for 4-chlorocatechol turnover (approximately 1% of the total recovered activity against 0.1 mM 4-chlorocatechol). Calculation of the metabolic flux of 0.1 mM 5-chlorosalicylate or 4-methylsalicylate in cells pregrown in each, based on the kinetic parameters obtained in this study or obtained previously (7) (Fig. 5), supported the notion that 5-chlorosalicylate degradation is driven predominantly by $C12O_{salD}$ and MCI_{ccaB} (95% and 81% of the overall flux in 5-chlorosalicylate-grown cells, respectively) and that $C120_{ccaB}$ is of minor importance. $C12O_{salD}$ and MCI_{salC} were of major importance for 4-methylsalicylate degradation (84% and 92% of the overall flux in


A
Actinobacterial catechol 1,2-dioxygenases
Proteobacterial catechol 1,2-dioxygenases
Chlorocatechol 1,2-dioxygenases
Hydroxybenzoquinol dioxygenases
CatA R. rhodochrous NCIMB 13259 AAC38303
CatA Rhodococcus sp. AN-22 BAE46387
CatA R. erythropolis AN13 BAA11859
CatA R. erythropolis CCM2595 CAE53841
CatA R. opacus 1CP CAA67941
Mflv 1358 M. gilvum PYR-GCK AB43840
CatA Arthrobacter sp. BA-5-17 BAD11154
CatA Arthrobacter sp. mA3 CAA03944
ClcA2 R. opacus 1CP CAD28142
CcaA P. reinekei MT1
ClcA R. opacus 1CP O67987
ClpC D. lusatiensis CAD60254
DccAI S. herbicidovorans MH CAF32818
DccAII S. herbicidovorans MH CAF32822
TfdC2 Sphingomonas sp. tfd44 AAT99373
TfdC Burkholderia sp. NK8 BAB56009
TfdCI C. necator JMP134 P05403
TfdC V. paradoxus AAC01745
TfdCII C. necator JMP134 U16782
ClcA P. putida AC 867 AAA98281
TetC P. chlororaphis RW71 CAB52140
TcbC Acidovorax sp. P51 P27098
PoaH P. putida ATCC 23975 AAB41024
HadC R. pickettii DTP0602 BAA13107
TcpC C. necator JMP134 AAM55216
CphAII A. chlorophenolicus AAN08760
DxnF S. wittichii RW1 CAA51371
PnpC Ralstonia sp. SJ98 AAS87586
PnpC Pseudomonas sp. PDS-7 ABW99096
HapG P. fluorescens ACB ACA50461
GraB Rhizobium sp. MTP-10005 BAF44523
CatA B. petrii YP 001630005
CatA A. baylyi ADP1 YP 046127
PheB Pseudomonas sp. EST1001 P31019
CatA1 Burkholderia sp. TH2 BAC16779
CatA1 Frateuria sp. ANA-18 BAA75207
CatA2 Burkholderia sp. TH2 BAC16769
CatA2 Frateuria sp. ANA-18 BAA75211
SalD P. reinekei MT1 ABI92974
CatA Pseudomonas sp. CA10 BAB32458
CatA P. aeruginosa PAO1 NP 251197
CatA P. putida PRS2000 U12557
CatA P. putida KT2440 NP 74584
0.1
B
β- and γ-Proteobacterial chloromuconate cycloisomerases
α-Proteobacterial chloromuconate cycloisomerases
Actinobacterial chloromuconate cycloisomerases
Actinobacterial muconate cycloisomerases
Proteobacterial muconate cycloisomerases
TcbD P. chlororaphis RW71 CAB88932
TcbD Acidovorax sp. P51 P27099
TfdD V. paradoxus TV1 BAA88065
ClcB P. putida AC867 P11452
TfdDI C. necator JMP134 P05404
TfdD Burkholderia sp. NK8 BAB56010
CatB2 Frateuria sp. ANA18 BAA75210
CatB2 Burkholderia sp. TH2 BAC16768
CatB A. baylyi ADP1 AAC46430
CatB B. petrii B. petrii YP 001630006
CatB1 Frateuria sp. ANA-18 BAA75205
CatB1 Burkholderia sp. TH2 BAC16777
CatB P. aeruginosa PAO1 NP 251199
CatB P. reinekei MT1 ABI 93945
SalC P. reinekei MT1 AAY23362
CatB Pseudomonas sp. CA10 BAB32456
CatB P. putida PRS2000 P08310
CatB P. putida KT2440 NP 74548
MdlA P. putida ATCC12633 P11444
TfdDII C. necator JMP134 U16782
CcaB P. reinekei MT1
CatB Rhodococcus sp. AN-22 BAE46388
CatB R. erythropolis AN13 BAC76455
CatB R. opacus 1CP P95608
CatB R. erythropolis CCM2595 CAE43842
ClcB R. opacus 1CP AAC38249
ClcB2 R. opacus 1CP CAD28144
TfdD Sphingomonas sp. tfd44 AAT99267
DccB S. herbicidovorans MH ABB00636
0.1

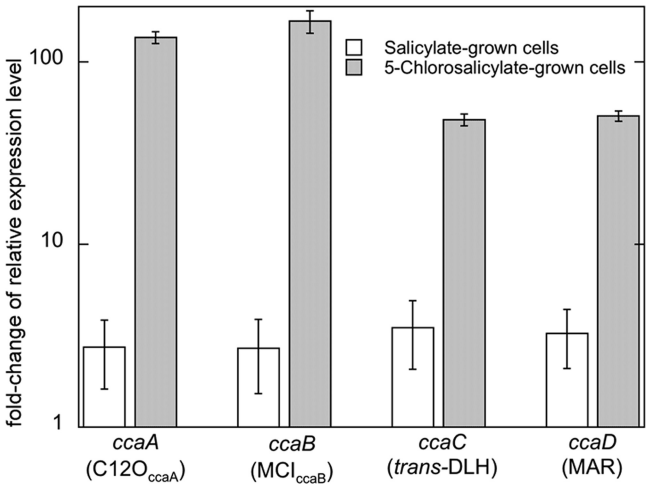


FIG. 4. Relative expression levels of catabolic genes in salicylate- and 5-chlorosalicylate-grown cells of *P. reinekei* MT1 as determined by quantitative RT-PCR. The values represent *n*-fold change (mean of triplicate samples) in the ratio of gene expression between the target gene and the reference gene (*rpsL*) compared to expression under noninducing conditions (for acetate-grown cells, this ratio was set at 1). The error bars indicate standard deviations.

4-methylsalicylate-grown cells). It should be noted, however, that the kinetic parameters used for these calculations reflect their activities in the enzymatic test and not necessarily their activities in situ.

DISCUSSION

Here, we report the identification of a set of five genes that are located in a 5.1-kb region of the genome of *P. reinekei* MT1 and that encode enzymes involved in the degradation of 5-chlorosalicylate via 4-chlorocatechol (Fig. 6).

In addition to the *ccaC* gene, encoding *trans*-DLH (8), this gene cluster comprised genes encoding functional $C12O_{ccaA}$ and MCI_{ccaB} proteins that were induced when the strain was grown on 5-chlorosalicylate (but also on 4-methylsalicylate). The presence of three distinct sets of (chloro)catechol 1,2-dioxygenases and (chloro)muconate cycloisomerases raises the question of their functions for the degradation of differently substituted salicylates in strain MT1. On one hand, the induction of $C12O_{ccaA}$ and MCI_{ccaB} during growth on chlorosalicylate indicates their involvement in the degradation of chloroaromatics. On the other hand, $C12O_{ccaA}$ was found to be only poorly active against 4-chlorocatechol, the central intermediate of chlorosalicylate degradation by MT1, and in its kinetic properties against catechol and 4-chlorocatechol, this enzyme resembles proteobacterial C12Os (6, 37, 49, 51). In contrast, $C12O_{salD}$, being coinduced during growth on chlorosalicylate, was reported to exhibit increased 4-chlorocatechol turnover rates compared with other proteobacterial C12Os (7). In fact, calculation of the relative activities against 4-chlorocatechol in cell extracts and of the metabolic flux indicated that $C12O_{salD}$, rather than $C12O_{ccaA}$, drives 4-chlorocatechol metabolism but indicated some importance of $C12O_{ccaA}$ for 4-methylcatechol metabolism.

The turnover of intermediate 4-chlorocatechol has been reported to be a pathway bottleneck for the growth of strain MT1 on chlorosalicylates (42), and at higher chlorosalicylate loads, 4-chlorocatechol was shown to accumulate. As chlorinated catechols are highly toxic to eukaryotic and bacterial cells (55), the concomitant accumulation of 4-chlorocatechol results in cell death and termination of degradative performance (43). The induction of two C12Os may result in a more robust degradative phenotype, avoiding to a significant extent the accumulation of 4-chlorocatechol. Accordingly, Perez-Pantoja et al. (43) showed that an efficient turnover of chlorocatechols is essential for the growth of *C. necator* JMP134 on 3-chlorobenzoate and that multiple copies of a chlorocatechol 1,2-dioxygenase gene are necessary to efficiently deplete chlorocatechols produced during 3-chlorobenzoate turnover by this strain. Taking into account the low turnover rate of both $C12O_{salD}$ and $C12O_{ccaA}$ for 4-chlorocatechol, it can be reasoned that their combined actions are necessary for efficient degradation.

P. reinekei MT1 was originally isolated from a four-member 4-chlorosalicylate-degrading bacterial community in which two other community members, namely, *Achromobacter spanius* MT3 and *Pseudomonas veronii* MT4 (41), were supposed to support degradation by depleting toxic metabolites, 4-chlorocatechol and protoanemonin, formed by MT1 during chlorosalicylate metabolism (42). Thus, it seems that MT1 is specifically adapted to degrade chlorosalicylates in concert with those strains due to rather ineffective chlorocatechol-transforming enzymes that are not suited for highly effective mineralization of chlorosalicylates in pure culture (41).

As for ring cleavage activities, two muconate-cycloisomerizing activities were also induced during growth of MT1 on chlorosalicylates. The major difference between these enzymes is the fact that MCI_{salC} predominantly catalyzes the formation of protoanemonin, a reaction that *trans*-DLH can interfere with to produce maleylacetate whereas MCI_{ccaB} catalyzes the transformation to approximately equal amounts of protoanemonin and *cis*-dienelactone. As *trans*-DLH cannot interfere with *cis*-dienelactone formation, MCI_{ccaB} can ensure a rapid metabolism of intermediate 3-chloromuconate but increases the formation of the *cis*-dienelactone dead-end intermediate. The presence of two MCIs assisting in the metabolism of chlorosalicylates may equip MT1 with a certain level of metabolic flexibility. Evidently, strain MT1 mineralizes 5-chlorosalicylate through a complex metabolic interplay between enzymes encoded by the *cca* and *sal* gene clusters.

Specific inactivation of genes of the *sal* and *cca* gene clusters will in future clarify their importance for the degradation of

FIG. 3. Dendrograms showing the relatedness of intradiol dioxygenases (A) and MCIs (B). The evolutionary history was inferred with MEGA4 (59) using the neighbor-joining algorithm 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. The scale bars indicate amino acid differences per site.

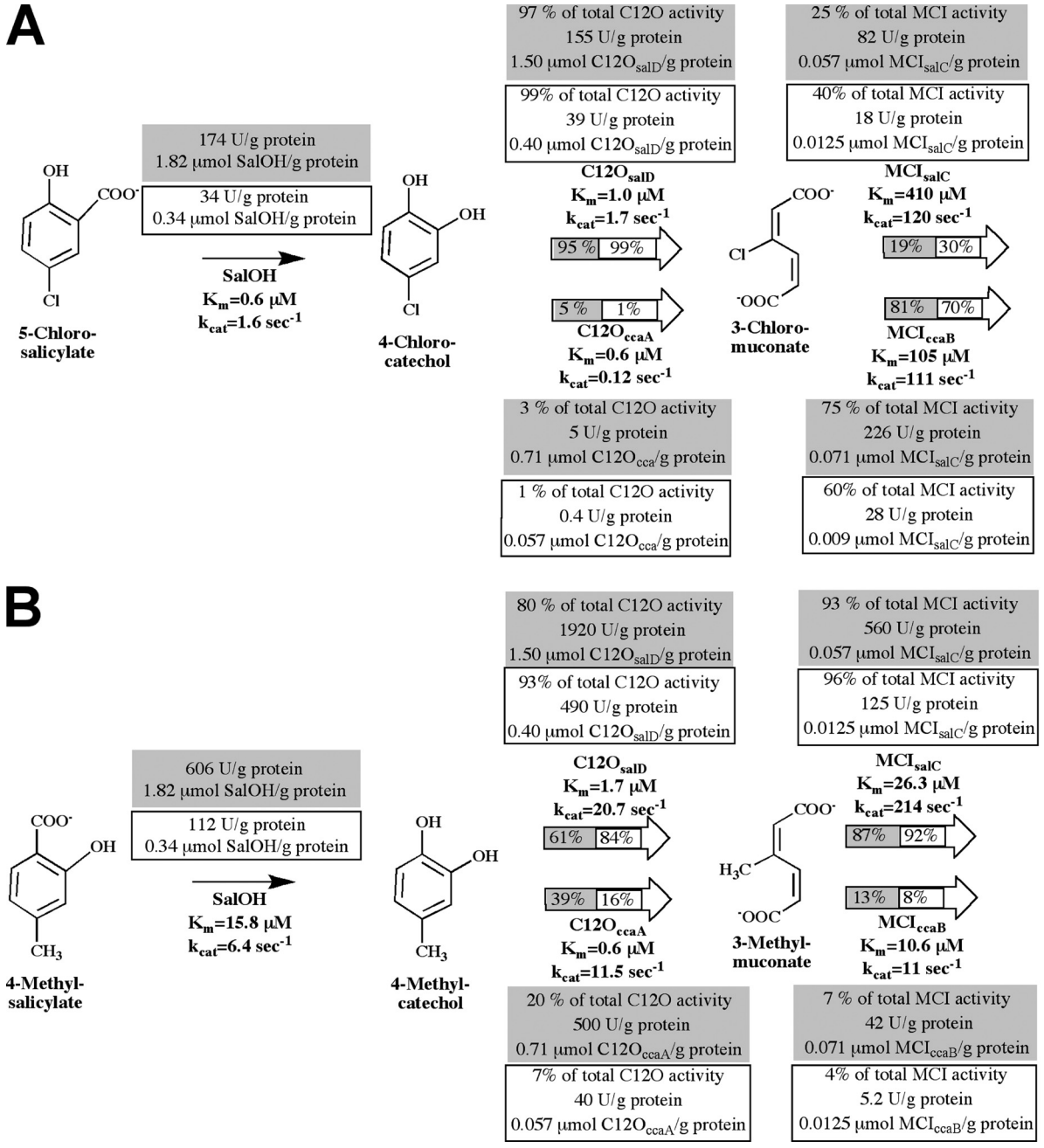


FIG. 5. Metabolism of 5-chlorosalicylate (A) or 4-methylsalicylate (B) by *P. reinekei* MT1. The kinetic constants of SalOH, $C12O_{salD}$, $C12O_{ccaA}$, MCI_{salC}, and MCI_{ccaB} are indicated. The specific activity (U/g protein) was determined in cell extracts, and the contribution of each of the (chloro)catechol 1,2-dioxygenases or (chloro)muconate cycloisomerases to the total activity against 0.1 mM 4-chlorocatechol or 0.1 mM 3-chloromuconate (A) or against 0.1 mM 4-methylcatechol or 0.1 mM 3-methylmuconate (B) in 5-chlorosalicylate-grown (gray) or 4-methylsalicylate-grown (boxed) cells was calculated after enzyme partial purification (given in percent and U/g protein). The enzyme concentrations (μmol/g protein) in the cell extracts were calculated based on the kinetic parameters of the enzyme of interest. The contributions of isoenzymes to the total metabolic flux of 0.1 mM 5-chlorosalicylate or 4-methylsalicylate by 5-chlorosalicylate-grown (gray) or 4-methylsalicylate-grown (boxed) cells were calculated by MATLAB and are given in percentages in the arrows.

chlorosalicylates by strain MT1 and the effects exerted when mutant MT1 strains have to interact with the above-described community members.

Two other catabolic enzymes are encoded in the *cca* gene cluster. The *ccaC* gene product (*trans*-DLH) has recently been described as a zinc-dependent hydrolase (8) that interacts with the cycloisomerization of 3-chloromuconate by hydrolyzing the intermediate 4-chloromuconolactone to maleylacetate (Fig. 6). The *ccaD* gene obviously encodes a MAR. Genes encoding MARs have initially been observed in chlorocatechol gene operons (28, 36, 56, 57), where the encoding enzymes catalyze a crucial degradation step channeling the substrate into the

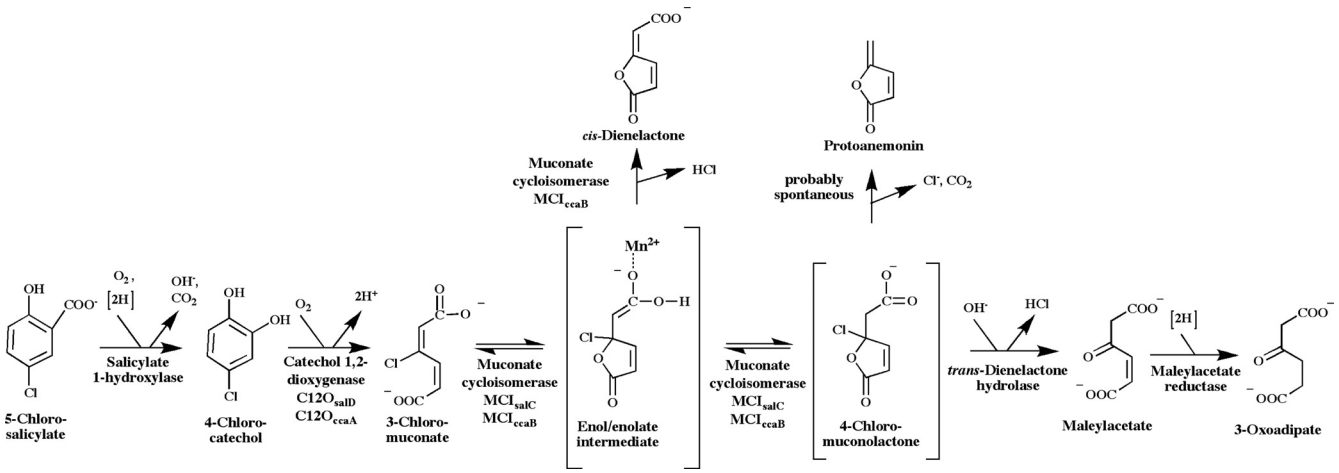


FIG. 6. Degradation of 5-chlorosalicylate by *P. reinekei* MT1. Designations of gene products are given below the reaction steps.

3-oxoadipate pathway (47). MARs are also involved in the degradation of chloroaromatics via hydroxybenzoquinols, such as in the degradation of 2,4,5-trichlorophenoxyacetate (25) or 2,4,6-trichlorophenol (34); in the degradation of sulfoaromatics (16, 21); and in the degradation of natural aromatics, such as resorcinol (9, 24).

The *cca* gene cluster of MT1 not only presents a novel gene arrangement, but specifically comprises enzymes only distantly related ($C12O_{ccaA}$ and MCI_{ccaB}) or completely unrelated (*trans*-DLH) to enzymes previously described as involved in catechol or chlorocatechol metabolism. Also unexpected was the observation of a gene encoding an IclR-type regulator transcribed divergently compared to the *ccaA* and *ccaB* genes, as catechol and chlorocatechol catabolic gene clusters are commonly under the control of a LysR-type regulator (62). Protocatechuate catabolic gene clusters, in contrast, are usually regulated by IclR-type regulators, such as PcaR of *P. putida* (50), PcaU of *Acinetobacter* sp. strain ADP1 (18), PcaR of *R. opacus* 1CP (14), and PcaQ of *Agrobacterium tumefaciens* (40). A gene organization similar to that in MT1 has so far been described only by Eulberg and Schlömann (15) for the *catABC* gene cluster from *R. opacus* 1CP, where a gene encoding an IclR-type regulator is transcribed divergently to a gene encoding C12O. However, in contrast to the observation by Eulberg and Schlömann, who argued that after the divergence of the *cat* genes found in *Rhodococcus* from other catechol genes the original LysR-type regulator gene was replaced by one belonging to the PobR subfamily of IclR-type regulators, no indications of the evolutionary events leading to the development of the MT1 *cca* cluster can be given at this time, as both $C12O_{cca}$ and MCI_{ccaB} seem to represent a new lineage in the phylogeny of intradiol dioxygenases.

It is astonishing that despite the tremendous efforts in sequencing isolates and in isolating new organisms with new catabolic properties, these new lineages have not yet been observed. One of the possible reasons may be the restricted substrate specificity for metabolism of specifically *p*-substituted catechols and *m*-substituted muconates. Specifically, the catabolic properties of MCI_{ccaB} deserve special attention, as it showed metabolic properties not yet reported for any cycloisomerase, producing both *cis*-dienelactone (as do chloromuconate cycloisomerases) and protoanemonin (as do MCIs) (4, 39, 53, 54, 58). Studies of the mechanism of MCI have suggested that the reaction proceeds via an enol/enolate to which a proton is added to form muconolactone (19), as depicted in Fig. 6. Similarly, the formation of protoanemonin from 3-chloro-*cis*,*cis*-muconate involves a protonation reaction, whereas in the reaction of chloromuconate cycloisomerases with 3-chloromuconate, the corresponding enol/enolate intermediate is not protonated but rather loses the negative charge by chloride abstraction (29). Replacement of Lys169 of *P. putida* PRS2000 MCI, which is known to provide the proton for the protonation reaction (19, 52), by alanine resulted in mutants that were not able to form protoanemonin but rather formed *cis*-dienelactone (29). However, as a protonating lysine residue is also conserved in chloromuconate cycloisomerases, as it is in MCI_{ccaB}, it was proposed that during the divergence of chloromuconate cycloisomerases from MCIs the rate of chloride elimination from the enol/enolate intermediate was enhanced, even though residues that could accelerate chloride elimination could not yet be identified in chloromuconate cycloisomerases (29). MCI_{ccaB} appears from the mechanistic and genetic points of view to be an evolutionary intermediate between chloromuconate cycloisomerases and MCIs, in which the rate of dechlorination was enhanced compared to those of MCIs (as was evident from the formation of *cis*-dienelactone) but significant rates of proton addition were also observed (as was evident from the formation of protoanemonin). Thus, a detailed analysis of the substrate binding pocket of MCI_{ccaB} could reveal important information about residues crucial for dehalogenation.

ACKNOWLEDGMENTS

The work was supported by the DFG-European Graduate College 653.

We thank Rita Getzlaff (HZI) for N-terminal protein amino acid sequencing. We gratefully acknowledge Iris Plumeier and Agnes Waliczek for their excellent technical support and Melissa Wos-Oxley for critical reading of the manuscript.

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