Characterization of a gene cluster involved in 4-chlorocatechol degradation by *Pseudomonas reinekei* MT1

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Pseudomonas reinekei MT1 has previously been described 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, C12OccaA, a novel (chloro)muconate cycloisomerase MCIccab was induced which showed features not yet reported. This cycloisomerase, being 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 encoding genes ccaA (C12OccaA) and ccaB (MCIccab) were located in a 5.1 kb genomic region clustered with genes encoding trans-dienelactone hydrolase (ccaC), maleylacetate reductase (ccaD) and a putative regulatory gene ccaR homologous to regulators of the IclR-type family. Thus this region encodes encoding genes sufficient to enable MT1 to transform 4-chlorocatechol to 3-oxoadipate. Phylogenetic analysis showed that C12OccaA and MCIccab are only distantly related to previously described catechol 1,2-dioxygenases and muconate cycloisomerases. Kinetic analysis indicated MCAccab and previously identified C12OsalD rather than C12OccaA to be crucial for 5-chlorosalicylate degradation. Thus, MT1 uses enzymes of 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.
INTRODUCTION

The aerobic degradation of chloroaromatic compounds usually proceeds via chlorocatechols as central intermediates (24, 47) which in most of the cases reported thus far, are further degraded by enzymes of the chlorocatechol pathway (44). This pathway involvesortho-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. *P. 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, which involve reactions known from the chlorocatechol as well as the 3-oxoadipate pathway, have recently been observed in *Rhodococcus opacus* 1CP (35) and *P. 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 to dehalogenate 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 intermediate (39). In contrast to all previously described diene lactone hydrolases involved in chlorocatechol degradation, which belong to the αβ hydrolase fold enzymes with a catalytic triad consisting of Cys, His and Asp (8), *trans*-DLH was shown to be a zinc-dependent hydrolase (10). The function of this enzyme in the 4-chlorocatechol metabolic pathway was to interact with the muconate cycloisomerase 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 (9) 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 being clustered with *salCD* genes encoding muconate cycloisomerase and catechol 1,2-dioxygenase, 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 muconate cycloisomerases 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 catechol 1,2-dioxygenase, trans-dienelactone hydrolase (10) and a maleylacetate reductase. Evidently, *P. reinekei* MT1 is the first microorganism where 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 prepared as previously described (39).

**Enzyme assays.** Catechol 1,2-dioxygenase (C12O), muconate cycloisomerase (MCI) trans-dienelactone hydrolase (trans-DLH) and maleylacetate reductase (MAR) activities were determined spectrophotometrically as previously described (27, 39, 54). Activity of MCI_{ccaB} with 3-chloromuconate was determined by HPLC (39) following substrate depletion and product formation. To more sensitively follow activity of MCI_{ccaB} with muconate and 2-chloromuconate, 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 protein standard (5).
**Analysis of kinetic data.** $V_{\text{max}}$, $k_{\text{cat}}$ and apparent $K_m$ values of C12O$_{\text{ccaA}}$ with catechol, 3-methylcatechol, 4-methylcatechol and 4-chlorocatechol were determined using 1 – 100 µM of substrate in air-saturated buffer and kinetic data were calculated from the initial velocities using the Michaelis-Menten equation by non-linear 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 (9). $V_{\text{max}}$, $k_{\text{cat}}$ and apparent $K_m$ values of MCI$_{\text{ccaB}}$ with 2-methyl-, and 3-methylmuconate were determined using 2 - 100 µM of substrate. Transformation of 3-chloromuconate was determined by HPLC analysis at substrate concentrations of 50 µM - 500 µM. Samples were taken during the reaction time and formation of protoanemonin and cis-dienelactone were directly quantified by HPLC analysis. At least two independent experiments were performed for each value. $K_m$ and $V_{\text{max}}$ values were calculated by nonlinear regression to the Michaelis-Menten equation, using KaleidaGraph (Synergy Software). Turnover numbers ($k_{\text{cat}}$ values) were calculated assuming subunit molecular masses of 29,424 (C12O$_{\text{ccaA}}$) and 39,764 (MCI$_{\text{ccaB}}$), respectively.

**Enzyme purification.** Catechol 1,2-dioxygenase (C12O$_{\text{ccaA}}$) and muconate cycloisomerase (MCI$_{\text{ccaB}}$) 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 Tris/HCl (50 mM, pH 7.5, 2 mM MnCl$_2$).

For analyzing the presence and abundance of different catechol 1,2-dioxygenases and muconate cycloisomerases under different growth conditions, cell extracts (usually containing 35 mg of protein per ml) were either directly applied onto a MonoQ HR 5/5 (Amersham Pharmacia Biotech) and proteins eluted by a linear gradient of 0 – 0.5 M NaCl over 25 ml
with a flow of 0.5 ml/min or the cell extract was mixed with 4 M (NH₄)₂SO₄ to give a final concentration of 1 M (NH₄)₂SO₄ 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 (NH₄)₂SO₄ (1 M – 0 M) over 25 ml with a flow of 0.5 ml/min. Fraction volumes were 0.5 ml. Hydrophobic interaction chromatography (HIC) separated C12OccaA (0.52 ± 0.02 M), C12OsalD (0.45 ± 0.04 M), C12OcatA (0.16 ± 0.04 M), MClccaB (0.25 ± 0.04 M), MClsalC (0.06 ± 0.06 M) and MClcatB (0.12 ± 0.06 M), thus excluding interference between their activities. During anion exchange chromatography C12OccaA eluted at 0.23 ± 0.01M NaCl, whereas MClccaB eluted at 0.37 ± 0.02M NaCl. Under these conditions C12OcatA and C12OsalD had been shown to coelute at 0.28 ± 0.02M NaCl whereas MClcatB and MClsalC coeluted at 0.24 ± 0.02M NaCl (9).

For purification of C12OccaA, 35 mg of protein from 5-chlorosalicylate grown cells was applied onto the MonoQ HR 5/5 (Amersham Pharmacia Biotech) and proteins were eluted as described above. Fractions containing C12OccaA activity were combined, supplemented with 4 M (NH₄)₂SO₄ to give a final concentration of 1 M (NH₄)₂SO₄ and loaded on a SOURCE 15PHE PE 4.6/100 (hydrophobic interaction) column (Amersham Pharmacia Biotech) as described above.

For purification of MClccaB, up to 400 mg of protein from 5-chlorosalicylate grown cells was applied onto a MonoQ HR 10/10 (Amersham Pharmacia Biotech). A stepwise gradient of 0 – 60 mM NaCl over 40 ml, 60 – 380 mM over 120 ml and 380 – 2000 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 - 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 (NH₄)₂SO₄.
to give a final concentration of 0.8 M (NH₄)₂SO₄ 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 Centricon YM-50 (Millipore). Further purification was achieved by gel filtration using a Suprose 12 HR10/10 column (Amersham Pharmacia Biotech). Proteins were eluted with Tris/HCl (50 mM, 2 mM MnCl₂ 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 10.5-11.5 ml) were applied onto a MonoQ HR5/5 (anionic exchange) column (Amersham Pharmacia Biotech) and proteins were eluted by a linear gradient of 0 – 0.4 M NaCl over 25 ml with a flow of 0.2 ml/min. Homogeneity was verified by SDS-PAGE. trans-DLH was purified as previously described (10).

**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 MnCl₂, pH 7.5) with 120 µM 3-chloromuconate as 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 - 1320 mU/ml (determined by the transformation of 50 µM trans-dienelactone), corresponding to 0.88 - 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-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad Miniprotein II as previously described (32) with acrylamide concentrations of 5 and 10 % w/v used for the concentrating and separating gels, respectively. The proteins were stained with Coomassie brilliant blue (Serva). PageRuler Protein Ladder (Fermentas) was used as a marker.

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

**Identification of the gene encoding muconate cycloisomerase MCI\textsubscript{ccaB} of strain MT1.** Part of the gene encoding MCI\textsubscript{ccaB} was amplified by PCR using degenerate primers NT1B: WSNCA\text{RGG\text{N}TT\text{Y}GT\text{N}\text{A}TCGG} and NTREV2A: AANW\text{SCAT}\text{N}\text{C\text{K}\text{D}\text{A}T}\text{N}\text{G}\text{G}CTG, which were designed based on the determined N-terminal protein sequence (underlined) SQGFVIG\text{R\text{V\text{L}}A\text{Q\text{R\text{D\text{I}P\text{F\text{S\text{Q\text{P\text{R\text{M\text{S\text{F\text{G}\text{G\text{L}}}}}}}}}}}}\text{TLD. 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 pGEM-T Easy vector (Promega), transformed into *E. coli* XL10-Gold (Stratagene) and inserts of 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\textsubscript{ccaB} was amplified by PCR using the primers MCIB1: GCAACCGCTGGATATACCTT and MCIBR2: GTRTCGCCRCTSGC\text{S\text{A\text{R\text{C\text{G\text{T}}}}}}CTCC, 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.
Touchdown PCR conditions included 10 cycles as described above, followed by 25 cycles of 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 corresponds to part of the gene encoding the 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 (9). The fosmid library was screened by PCR using primers specific for ccaB (MCIB: GCAACGGCTGGATATACCTT and inMCIB: AGCAGAAACACCCAACTGCT, annealing temperature of 59°C). Fosmid clones harboring the expected 340 bp ccaB gene fragment were subsequently checked for the presence of the ccaC gene encoding trans-DLH by PCR (TransFOR: AATCCCTGCGACATACAAG and TransREV: CGTCAGCATGAAGGTGTAGC). Of the three fosmids carrying both the ccaB and the ccaC gene fragment, one fosmid was chosen, purified with the FosmidMAX DNA purification kit (Epicentre) and the complete cca gene cluster was obtained by direct sequencing (Seqlab, Göttingen) from the purified fosmid with a six-fold 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 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 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 (N-J) 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 nucleotide sequence reported in this study was deposited in the DDBJ/EMBL/GenBank databases under the accession number EF159980.

Gene expression studies. Harvest of *P. reinekei* MT1 cells and RNA extraction were done as previously described (9). Reverse transcription and quantitative real-time PCR were performed using QuantiTect SYBR green reverse transcription-PCR (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 (GGGCCTTTTCACACCAATGACC), *CcaA*-R (GCAGGTGAGCGGGTCGGAAGTA), *CcaB*-F (GCAGTTGAGGCCGCGGTTGTTA), *CcaB*-R (GCTTGCCAACCAGGGTGTA), *CcaC*-F (TGACACGTCCAAAATCCCTGCG) and *CcaC*-R (GCAAGCGTGCGGCTATCAAT), *CcaD*-F (GATGGCGTTGTCGGTCTTGG) and *CcaD*-R (TGACGTTTCAGGGCGGATA). A housekeeping reference gene (ribosomal *rpsL*) was selected to normalize the results obtained (9, 13). Real-time PCRs and relative expression ratios were carried out as previously described (9).

Mathematical calculations. Numerical calculations were performed with a kinetic model build in SIMULINK v6.4.1 under the MATLAB v7.2.0.232 environment (The MathWorks, Inc., Natick, Mas.) based on Michaelis-Menten kinetics using the kinetic constants experimentally determined here or previously (9) and assuming a constant concentration of enzyme and zero order kinetics for oxygen and NADH.
**Analytical methods.** High-performance liquid chromatography (HPLC) was performed as previously described (9).

**Chemicals.** 3-Chloro-, 4-chloro-, 3-methyl- and 4-methylcatechol were obtained from Helix Biotech (USA). 2-Methyl-, 3-methyl- and 3-chloro-\(cis, cis\)-muconate were freshly prepared from 3-methyl-, 4-methyl- and 4-chlorocatechol, respectively, in Tris/HCl (50 mM, pH 7.5, 2mM MnCl\(_2\)) using chlorocatechol 1,2-dioxygenase TetC of *P. chlororaphis* RW71 (45) or partially purified C12O\(_{aal}\) 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).

**RESULTS**

**Characterization of a cycloisomerase transforming 3-chloromuconate into both \(cis\)-dienelactone and protoanemonin.** Two muconate cycloisomerases, both transforming 3-chloromuconate into protoanemonin, besides minor quantities of \(cis\)-dienelactone had previously been characterized from *P. reinekei* MT1 and the encoding genes had been localized (9). However, during growth on 5-chlorosalicylate, the presence of a distinct enzyme capable of transforming 3-chloromuconate was evident. This enzyme, termed MCI\(_{cabc}\) 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 product mixture was not previously observed by any muconate or chloromuconate...
cycloisomerase, the respective enzyme was purified to homogeneity. The native molecular mass of MCI\textsubscript{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\textsubscript{ccaB}, like muconate cycloisomerase of \textit{P. putida} PRS2000 (21) or chloromuconate cycloisomerase from \textit{C. necator} JMP 134 (22), may be a homo-octamer. N-terminal amino acid analysis (SQGFVIGRVLAQLQRLDIPFSQPIRMSFGTLD) revealed no significant similarity when these sequences were compared to 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, specificity constants of 3-chloro- 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\textsubscript{ccaB} is clearly different from previously reported muconate cycloisomerases, which are characterized by their high activity with muconate (53, 54). It also differed from MCI\textsubscript{salC} of MT1, which has previously been characterized to be adapted for the turnover of 3-methylmuconate (9) but retained a significant activity with muconate. MCI\textsubscript{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\textsubscript{ccaB} transformed 3-chloromuconate stoichiometrically into equal amounts of protoanemonin and \textit{cis}-dienelactone contrasts all previously described cycloisomerases, which form either protoanemonin (muconate cycloisomerases) or \textit{cis}-dienelactone as the predominant product (chloromuconate cycloisomerases) (4, 39, 53, 54,
Following 3-chloromuconate transformation over time showed that both products were formed in 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\textsubscript{salC} (39), an enzyme encoded by the \textit{sal} cluster and induced during growth on chlorosalicylates (9), and it was suggested that trans-DLH acts on intermediate 4-chloromuconolactone to form maleylacetate thereby preventing protoanemonin formation. To validate that trans-DLH can similarly interact with MCI\textsubscript{ccaB}, 3-chloromuconate (0.12 mM) was transformed by enzyme mixtures comprising MCI\textsubscript{ccaB} (8.8 nM) and varying amounts of trans-DLH (0.88 – 88 nM). As previously observed for MCI\textsubscript{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 substrate transformed.

**Characterization of a catechol 1,2-dioxygenase specifically induced during growth on 5-chlorosalicylate.** As during growth on chlorosalicylate a muconate cycloisomerase was induced, which was not encoded by the previously described \textit{cat} or \textit{sal} gene clusters (9), it was assessed whether a distinct catechol 1,2-dioxygenase was also induced under such conditions. In fact, catechol 1,2-dioxygenase activity was observed in protein fractions of cell extracts, eluting at 0.23 ± 0.01 M NaCl during anionic exchange chromatography, beside previously described C12O\textsubscript{salC}, eluting at 0.29 ± 0.01 M NaCl. HIC confirmed the presence of a previously uncharacterized catechol dioxygenase, termed C12O\textsubscript{ccaA} eluting at 0.52 ± 0.02 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} in 5-chlorosalicylate grown cells.

C12O\textsubscript{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 (AVSRLAELVTALES), showed no significant similarity with any proteins in public databases. It thus seems that C12O\textsubscript{ccaA} is only distantly related to previously characterized catechol 1,2-dioxygenases.

Kinetic data were measured directly in fractions, comprising C12O\textsubscript{ccaA} with a purity of at least 95\% of total protein. Thus it can be calculated that maximum turnover rates with catechol of 2375 U/g of protein correspond to activities of 2500 ± 100 U/g C12O\textsubscript{ccaA} and, based on a subunit molecular mass of 29.424 kDa (as supposed for the predicted amino acid sequence of C12O\textsubscript{ccaA}, see below), to a k\textsubscript{cat} value for catechol of 1.2 ± 0.05 s\(^{-1}\) (Table 1). This was approximately one order of magnitude lower than those previously reported for C12O\textsubscript{catA} and C12O\textsubscript{salD} and of other previously analyzed proteobacterial catechol 1,2-dioxygenases (6, 37, 49, 51). A high turnover rate was observed only for 4-methylcatechol, and a comparison of specificity constants k\textsubscript{cat}/K\textsubscript{m} showed 4-methylcatechol to be the highly preferred substrate (Table 1). A similar substrate profile has so far only been observed for C12O\textsubscript{salD} and contrasts that reported for either catechol or chlorocatechol 1,2-dioxygenases (3, 6, 11, 45). However, the degree of specificity of C12O\textsubscript{ccaA} was even more remarkable than that of C12O\textsubscript{salD}, as specificity constants for 4-methylcatechol compared to those for catechol, 4-chlorocatechol and 3-methylcatechol differed by a factor of 30-100. Surprisingly, activity of C12O\textsubscript{ccaA} against 4-chlorocatechol was rather poor and similar to that of previously described catechol 1,2-dioxygenases (11, 30, 38, 51).

**Characterization of the \textit{cca} gene cluster.** To localize genes encoding C12O\textsubscript{ccaA} and MCI\textsubscript{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 MCI\textsubscript{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 approx. 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 \textit{trans}-DLH (10) showed that both genes were encoded on the same fosmid, which contained an approx. 37.6 kb DNA fragment from MT1.

Sequencing of the insert revealed an approx. 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 \textit{ccaB}, contained the above identified 400 bp fragment encoding part of MCI_{ccaB} and can thus be supposed to encode MCI_{ccaB}. The \textit{ccaB} gene product showed only moderate identity to proteobacterial muconate cycloisomerase (35 - 42%), proteobacterial chloromuconate cycloisomerase (33 - 40%) or muconate and chloromuconate cycloisomerases identified from Gram+ microorganisms (35 - 37%), which in a phylogenetic analysis, form separate branches with low sequence identity to one another (see Fig. 3). This indicated that MCI_{ccaB} of strain MT1 forms a new branch illustrating a distinct evolutionary history. Upstream of \textit{ccaB}, \textit{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 catechol 1,2-dioxygenases (30-38%), proteobacterial chlorocatechol 1,2-dioxygenases (32-37%) or catechol and chlorocatechol 1,2-dioxygenases from Gram+ microorganisms (31-43%). Lower sequence identity (27-33%) was observed with members of the hydroxyquinol branch of intradiol dioxygenases (1, 17). The predicted amino acid sequence of the ORF transcribed divergently towards \textit{ccaA} and designated \textit{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). 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 of identified function (40% of 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 (10) and designated *ccaC* could be localized. The deduced product of the downstream *ccaD* gene showed highest sequence homology with maleylacetate reductases, with highest identity (59%) being observed with maleylacetate reductase TfdF2 of the 2,4-D degrading *Sphingomonas* sp. TFD44 (60).

**RT-PCR analysis of the cca cluster.** Accumulation of transcripts of *ccaA*, *ccaB*, *ccaC* and *ccaD* was measured during growth on 5-chlorosalicylate, salicylate and acetate (non-inducing 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 (50- to 150-fold) but not in salicylate-grown cells (Fig. 4).

**Induction of C12O*<sub>ccaA</sub>* and MCI*<sub>ccaB</sub>* 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 respectively encoded catechol 1,2-dioxygenases and muconate cycloisomerases was assessed after growth on 5-chlorosalicylate and 4-methylsalicylate. Cell extracts were separated by anionic exchange chromatography, fractions
monitored for transformation of 4-methylcatechol and 3-methylmuconate and activities quantified. Both catechol 1,2-dioxygenase as well as muconate cycloisomerizing activities could be nearly quantitatively recovered (recovery was >90 % for catechol 1,2-dioxygenase activity against 4-methylcatechol, and 85 – 95 % for muconate cycloisomerase activity against 3-methylmuconate).

Fractions of cell extracts of 5-chlorosalicylate grown cells eluting at 0.23 ± 0.01 M NaCl and thus containing C12OccaA 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 C12OsalD accounted for 80 ± 5 % of the total activity against 0.1 mM 4-methylcatechol (see 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 C12OccaA. Similar results were obtained when analyzing activities against 0.1 mM 3-methylmuconate 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 to be due to MCCIcab. This indicated C12OccaA and MCCIcab to be of only minor importance during degradation of 4-methylsalicylate. In contrast, a calculation of the respective activities against 0.1 mM 3-chloromuconate indicate 75 % ± 5 of the total activity in extracts of 5-chlorosalicylate grown cells to be due to induction of MCCIcab, whereas C12OccaA seems to be of minor importance for 4-chlorocatechol turnover (approx. 1 % of total recovered activity against 0.1 mM 4-chlorocatechol). Calculation of the metabolic flux of 0.1 mM 5-chlorosalicylate or 4-methylsalicylate in respectively pre-grown cells based on the kinetic parameters obtained in this study or obtained previously (9) (see Fig. 5) supported the notion of 5-chlorosalicylate degradation to be driven predominantly by C12OsalD and MCCIcab (95 % and 81 % of the overall flux in 5-chlorosalicylate-grown cells, respectively) and C12Occiab to be of minor importance. C12OsalD and MCCIcab were of major
importance for 4-methylsalicylate degradation (84% and 92% of the overall flux in 4-
methylsalicylate-grown cells). It should, however, be noted, that kinetic parameters used for
these calculations reflect their activity in the enzymatic test, and not necessarily their activity
in-situ.

Discussion

Here we report the identification of a set of five genes which 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 *trans*-DLH encoded by the *ccaC* gene (10), this gene cluster comprised
genes encoding functional C12O_{ccaA} and MCI_{ccaB} proteins which 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 function for the degradation of differently substituted salicylates in
strain MT1. On the 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, C12O_{ccaA} was found to be only poorly active against 4-chlorocatechol, the central
intermediate of chlorosalicylate degradation by MT1, and regarding its kinetic properties
against catechol and 4-chlorocatechol, this enzyme resembles proteobacterial catechol 1,2-
dioxygenases (6, 37, 49, 51). In contrast, C12O_{salD} being co-induced during growth on
chlorosalicylate was reported to exhibit increased 4-chlorocatechol turnover rates when
compared with other proteobacterial catechol 1,2-dioxygenases (9). In fact, calculation of the
relative activities against 4-chlorocatechol in cell extracts and of the metabolic flux indicated
C12O_{salD} rather than C12O_{ccaA} to drive 4-chlorocatechol metabolism, but indicated some importance of C12O_{ccaA} for 4-methylcatechol metabolism.

The turnover of intermediate 4-chlorocatechol has already previously been indicated to be a pathway bottleneck for growth of strain MT1 on chlorosalicylates (42) and at higher chlorosalicylate load, 4-chlorocatechol was shown to accumulate. As chlorinated catechols are highly toxic to eukaryotic and bacterial cells (55), the concomitant accumulation of 4-chlorocatechol will result in cell death and depletion of degradative performance (43). The induction of two catechol 1,2-dioxygenases may result in a more robust degradative phenotype, avoiding to a significant extent the accumulation of 4-chlorocatechol.

Accordingly, Perez-Pantoja et al. (43) had shown that an efficient turnover of chlorocatechols is essential for 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 action is necessary for an efficient degradation.

*P. reinekei* MT1 was originally isolated from a 4-membered 4-chlorosalicylate degrading bacterial community, where two other community members, namely *Achromobacter spanius* MT3 and *Pseudomonas veronii* MT4 (41) were supposed to support degradation by depleting toxic metabolites formed by MT1 during chlorosalicylate metabolism, namely 4-chlorocatechol and protoanemonin (42). It thus seems that MT1 is specifically adapted to degrade chlorosalicylates in concert with those strains due to rather ineffective chlorocatechol transforming enzymes not suited for highly effective mineralization of chlorosalicylates in pure culture (41).
Like for ring-cleavage activities, also two muconate cycloisomerizing activities were induced during growth of MT1 on chlorosalicylates. The major difference in these enzymes is the fact that MCI \textit{salC} catalyzes predominantly the formation of protoanemonin, a reaction where \textit{trans-DLH} can interfere with to produce maleylacetate whereas MCI \textit{ccaB} catalyzes the transformation to approximately equal amounts of protoanemonin and \textit{cis-dienelactone}. As \textit{trans-DLH} cannot interfere with \textit{cis-dienelactone} formation, MCI \textit{ccaB} can ensure a rapid metabolism of intermediate 3-chloromuconate, but increases the formation of the \textit{cis-dienelactone} dead-end intermediate. The presence of two muconate cycloisomerases assisting in the metabolism of chlorosalicylates may equip MT1 with a certain level of metabolic flexibility. Evidently, strain MT1 mineralizes 5-chlorosalicylate by a complex metabolic interplay between enzymes encoded by the \textit{cca} and \textit{sal} gene clusters.

Specific inactivation of genes of the \textit{sal} and \textit{cca} gene cluster will in future clarify their importance for degradation of 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 \textit{cca} gene cluster. The \textit{ccaC} gene product (\textit{trans-DLH}) has recently been described as a zinc-dependent hydrolase (10), which interacts with the cycloisomerization of 3-chloromuconate by hydrolyzing intermediate 4-chloromuconolactone to maleylacetate (Fig. 6). The \textit{ccaD} gene obviously encodes a maleylacetate reductase. Genes encoding maleylacetate reductases 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 3-oxoadipate pathway (47). Maleylacetate reductases 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, 20) and in the
degradation of natural aromatics such as resorcinol (7, 23).

The cca gene cluster of MT1 presents not only a novel gene arrangement, but specifically
comprises enzymes only distantly related (C12O$_{ccaA}$, MCI$_{ccaB}$) or completely unrelated (trans-
DLH) to enzymes previously described to be 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 Rhodococcus opacus 1CP (14) and PcaQ of Agrobacterium tumefaciens (40). A gene
organization similar to that in MT1 has so far been only described by Eulberg and Schlömann
(15) for the catABC gene cluster from Rhodococcus opacus 1CP, where a gene encoding a
IclR type regulator is transcribed divergently to a gene encoding catechol 1,2-dioxygenase.
However, in contrast to the observation by Eulberg and Schlömann, who argued that after
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 on the evolutionary events leading to the development of the MT1
cca cluster can be given as 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 sequencing efforts on isolates, and the
tremendous efforts on 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 \text{ccaB} deserve special attention as it showed metabolic properties not yet reported for any cycloisomerase thus far, producing both cis-dienelactone (as do chloromuconate cycloisomerases) and protoanemonin (as do muconate cycloisomerases) (4, 39, 53, 54, 58). Studies on the mechanism of muconate cycloisomerase 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-\textit{cis},\textit{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 \textit{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 \textit{cis}-dienelactone (29). However, as a protonating lysine residue is also conserved in chloromuconate cycloisomerases, like it is in MCI \text{ccaB}, it was proposed that during the divergence of chloromuconate cycloisomerases from muconate cycloisomerases the rate of chloride elimination from the enol/enolate intermediate was enhanced, even though residues which could accelerate chloride elimination could not yet be identified in chloromuconate cycloisomerases (29). MCI \text{ccaB} appears from the mechanistic and genetic point of view to be an evolutionary intermediate between chloromuconate cycloisomerases and muconate cycloisomerases, where the rate of dechlorination was enhanced compared to muconate cycloisomerases (as evident by the formation of \textit{cis}-dienelactone), but also significant rates of proton addition were observed (as evident by the formation of protoanemonin). Thus a detailed analysis of the substrate binding pocket of MCI \text{ccaB} could reveal important information on residues crucial for dehalogenation.
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LEGENDS TO FIGURES

Fig. 1. Ratio of maleylacetate and protoanemonin formed from 3-chloromuconate by mixtures of muconate cycloisomerase MCI$_{ccaB}$ (8.8 nM) with varying amounts of trans-DLH (0 – 88 nM) of \textit{P. reinekei} MT1. Reaction mixtures contained Tris/HCl (50 mM, 2 mM MnCl$_2$, pH 7.5) and 120 µM 3-chloromuconate. Substrate and product concentrations were analyzed by HPLC.

Fig. 2. Gene organization of a 5129-bp region from \textit{P. reinekei} MT1 containing the cca gene cluster. Arrows indicate gene orientations: cca$_A$, catechol 1,2-dioxygenase gene; cca$_B$, muconate cycloisomerase gene; cca$_C$, trans-dienelactone hydrolase gene; cca$_D$, putative maleylacetate reductase gene and cca$_R$, putative transcriptional regulator gene. Abbreviations of encoded enzymes are given below the gene clusters.

Fig. 3. Dendrograms showing the relatedness of intradiol dioxygenases (A) and muconate cycloisomerases (B). The evolutionary history was inferred with MEGA4 (59) using the Neighbor-Joining (N-J) 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 bar indicates amino acid differences per site.

Fig. 4. Relative expression levels of catabolic genes in salicylate and 5-chlorosalicylate-grown cells of \textit{P. reinekei} MT1 as determined by quantitative RT-PCR. 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 noninducing conditions (for acetate-grown cells, this ratio was set at 1).

**Fig. 5.** Metabolism of 5-chlorosalicylate (A) or 4-methylsalicylate (B) by *P. reinkei* MT1. 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 free extracts and the contribution of each of the (chloro)catechol 1,2-dioxygenases or (chloro)muconate cycloisomerasases 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 (grey) or 4-methylsalicylate-grown cells (boxed) calculated after enzyme partial purification (given in % and U/g protein). The enzyme concentration (µmol/g protein) in respective cell extracts was calculated based on kinetic parameters of the enzyme of interest. The contribution of isoenzymes to the total metabolic flux of 0.1 mM 5-chlorosalicylate or 4-methylsalicylate by 5-chlorosalicylate- (grey) or 4-methylsalicylate-grown cells (boxed) was calculated by MATLAB and is given in % in the hollow arrows.

**Fig. 6** Degradation of 5-chlorosalicylate by *P. reinkei* MT1. Designations of gene products are given below the reaction step.
Table 1. Substrate specificities of catechol 1,2-dioxygenase C12O_{caA} and muconate cycloisomerase MCI_{caB} from \textit{P. reinekei} MT1\textsuperscript{a}

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

\textsuperscript{a} The kinetic constants were determined as described in Materials and Methods. Standard deviations were calculated with the KaleidaGraph program. ND, not determined.
A

B

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