Characterization of the genes encoding the 3-carboxy-*cis,cis*-muconate-lactonizing enzymes from the 4-sulfocatechol degradative pathways of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2

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Hydrogenophaga intermedia strain S1 and *Agrobacterium radiobacter* strain S2 form a mixed bacterial culture which degrades sulfanilate (4-aminobenzenesulfonate) by a novel variation of the β -ketoadipate pathway via 4-sulfocatechol and 3-sulfomuconate. It was previously proposed that the further metabolism of 3-sulfomuconate is catalysed by modified 3-carboxy-*cis,cis*-muconate-lactonizing enzymes (CMLEs) and that these 'type 2' enzymes were different from the conventional CMLEs ('type 1') from the protocatechuate pathway in their ability to convert 3-sulfomuconate in addition to 3-carboxy-*cis,cis*-muconate. In the present study the genes for two CMLEs (*pcaB2S1* and *pcaB2S2*) were cloned from *H. intermedia* S1 and *A. radiobacter* S2, respectively. In both strains, these genes were located close to the previously identified genes encoding the 4-sulfocatechol-converting enzymes. The gene products of *pcaB2S1* and *pcaB2S2* were therefore tentatively identified as type 2 enzymes involved in the metabolism of 3-sulfomuconate. The genes were functionally expressed and the gene products were shown to convert 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate. 4-Carboxymethylene-4-sulfo-but-2-en-olide (4-sulfomuconolactone) was identified by HPLC-MS as the product, which was enzymically formed from 3-sulfomuconate. His-tagged variants of both CMLEs were purified and compared with the CMLE from the protocatechuate pathway of *Pseudomonas putida* PRS2000 for the conversion of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate. The CMLEs from the 4-sulfocatechol pathway converted 3-sulfomuconate with considerably higher activities than 3-carboxy-*cis,cis*-muconate. Also the CMLE from *P. putida* converted 3-sulfomuconate, but this enzyme demonstrated a clear preference for 3-carboxy-*cis,cis*-muconate as substrate. Thus it was demonstrated that in the 4-sulfocatechol pathway, distinct CMLEs are formed, which are specifically adapted for the preferred conversion of sulfonated substrates.

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INTRODUCTION

Aromatic compounds which carry sulfonic acid substituents directly attached to the aromatic ring system are extremely

rare among natural compounds, but are produced in large quantities by the chemical industry as detergents, dispersants, dyes, optical brighteners, ion exchangers and pharmaceuticals (Tully, 1997). Laboratory studies have shown that a sulfonic acid substituent usually significantly decreases the rates of biodegradation, and several examples of the accumulation of sulfonated aromatics in the environment have been described (Alexander & Lustigman, 1966; Alonso *et al.*, 1999; Knepper, 2002; Riediker *et al.*, 2000; Ruckstuhl *et al.*, 2002; Wellens, 1990). The microbial degradation of aromatic sulfonic acids has mainly been studied using simple benzenesulfonates and naphthalenesulfonates as model compounds. These studies demonstrated that sulfonated substrates are in most cases initially desulfonated by the action of ring-hydroxylating dioxygenases to the corresponding diols (Brilon *et al.*, 1981;

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Abbreviations: CMLE, 3-carboxy-*cis,cis*-muconate-lactonizing enzyme; NCBI, National Center for Biotechnology Information; P34OII, type 2 protocatechuate 3,4-dioxygenase.

The GenBank/EMBL/DDBJ accession numbers for the *pcaB2S2* and *pcaB2S1* sequences reported in this paper are AY769867 and AY769868, respectively.

Cook *et al.*, 1999; Nörtemann *et al.*, 1986; Ohe *et al.*, 1990; Thurnheer *et al.*, 1990; Wittich *et al.*, 1988). In contrast, it has been found that 4-aminobenzenesulfonate (sulfanilate) was initially deaminated by a co-culture of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 to 4-sulfocatechol (Feigel & Knackmuss, 1988). More recently, 4-sulfocatechol has also been described as an intermediate formed during the degradation of 1,3-benzenedisulfonate and linear alkylbenzenesulfonates (Contzen *et al.*, 1996; Schulz *et al.*, 2000; Dong *et al.*, 2004; Schleheck *et al.*, 2004). 4-Sulfocatechol thus appears to be a central intermediate for the degradation of substituted sulfonated benzenes, which are released by humans in enormous quantities ($>10^6$ tonnes annually) into the environment.

It was demonstrated for the sulfanilate-degrading mixed culture that 4-sulfocatechol was oxidized to 3-sulfomuconate by specifically adapted forms of protocatechuate 3,4-dioxygenases. These 'type 2' enzymes were therefore distinguished from the classical protocatechuate 3,4-dioxygenases ('type 1') which convert only protocatechuate (Feigel & Knackmuss, 1993; Hammer *et al.*, 1996). The genes encoding type 2 protocatechuate 3,4-dioxygenases (P34OIIs) have been cloned from *H. intermedia* S1 and *A. radiobacter* S2, and significant sequence similarities have been found among the genes encoding the type 1 and type 2 enzymes (Contzen & Stolz, 2000; Contzen *et al.*, 2001).

It has been suggested that 3-sulfomuconate is further converted in a cycloisomerization reaction to a sulfonated lactone (Fig. 1). This reaction is analogous to the cycloisomerization of 3-carboxy-*cis,cis*-muconate to 4-carboxymuconolactone found in the protocatechuate branch of the β -ketoadipate pathway, which is catalysed by 3-carboxy-*cis,cis*-muconate-lactonizing enzymes (CMLEs). The CMLE from *Pseudomonas putida* has been purified and the stereochemistry of the turnover of 3-carboxy-*cis,cis*-muconate has been analysed in some detail. In addition, the gene has been cloned and sequenced, and the crystal structure of the enzyme has been determined (Ornston, 1966; Williams *et al.*, 1992; Yang *et al.*, 2004).

In the present study, 3-sulfomuconate was prepared and the enzymes responsible for the metabolism of 3-sulfomuconate were characterized. The aim of the study was a further analysis of the enzymic and evolutionary relationship between the degradative pathways for protocatechuate and 4-sulfocatechol. The central questions in this context were if the 3-sulfomuconate-converting enzymes were indeed derived from 3-carboxy-*cis,cis*-muconate-converting enzymes, and if indications for a specific adaptation of these enzymes for the conversion of sulfonated substrates could be found.

METHODS

Bacterial strains and media. The isolation and characterization of *Hydrogenophaga intermedia* S1 (DSMZ 5680) and *Agrobacterium radiobacter* S2 (DSMZ 5681) have been reported before (Contzen

Fig. 1. Proposed pathway for the degradation of 4-sulfocatechol and protocatechuate by *A. radiobacter* S2 (Feigel & Knackmuss, 1993). Key to enzymes: I, P34OII; II, CMLE type 2; III, sulfomuconolactone hydrolase; IV, maleylacetate reductase; V, protocatechuate 3,4-dioxygenase type 1; VI, CMLE type 1; VII, γ -carboxymuconolactone decarboxylase; VIII, β -ketoadipate enol-lactone hydrolase. Key to compounds: 4SC, 4-sulfocatechol; 3SM, 3-sulfomuconate; 4SL, 4-sulfomuconolactone (4-carboxymethylene-4-sulfo-but-2-en-olide); MA, maleylacetate; KA, β -ketoadipate; PC, protocatechuate; 3CM, 3-carboxy-*cis,cis*-muconate; 4CL, carboxymuconolactone; EL, β -ketoadipate enol-lactone.

et al., 2000; Feigel & Knackmuss, 1988, 1993). The strains were routinely cultivated separately in SHPG-medium as described elsewhere (Contzen *et al.*, 2000; Feigel & Knackmuss, 1988, 1993).

Pseudomonas putida PRS2000 (Hughes *et al.*, 1988) was kindly provided by J. Gröning and M. Schlömann (TU Freiberg, Germany). The organism was grown at 30 °C on a rotary shaker (100 r.p.m.) in a mineral medium according to Dorn *et al.* (1974) with protocatechuate (7.5 mM) as sole source of carbon and energy. For isolation of genomic DNA, the strain was cultivated in liquid culture with NB [8 g dehydrated Nutrient Broth powder (Difco) and 5 g NaCl l⁻¹].

Escherichia coli DH5 α , JM 109, BL21(DE3)(pLysS) and BL21(DE3)(pLysS) Star (Invitrogen) were used as host strains for

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pJOE3075	Expression plasmid with a rhamnose-dependent promoter	Stumpp <i>et al.</i> (2000)
pAC28	Expression plasmid with the T7 promoter	Kholod & Mustelin (2001)
pETS2-X-II	Expression of <i>pcaH2G2</i> from <i>A. radiobacter</i> S2 under the control of the T7 promoter	Contzen <i>et al.</i> (2001)
pMCS2-2	<i>pcaG2</i> , genes for a putative TRAP transport system, putative IclR regulator and <i>pcaB2S2</i> from <i>A. radiobacter</i> S2 in pBluescript II SK(+)	Contzen & Stolz (2000)
pSHCMC2S2	<i>pcaB2S2</i> from <i>A. radiobacter</i> S2 in pAC28 (encodes ArCMLE2 with an amino-terminal His-tag)	This study
pSBCMC2S1	<i>pcaB2S1</i> from <i>H. intermedia</i> S1 in pJOE3075	This study
pSHCMC2S1	<i>pcaB2S1</i> from <i>H. intermedia</i> S1 in pAC28 (encodes HiCMLE2 with an amino-terminal His-tag)	This study
pSHPpCMLE	<i>pcaB</i> from <i>P. putida</i> PRS2000 in pAC28	This study

recombinant DNA work. *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium supplied with ampicillin ($100 \mu\text{g ml}^{-1}$) or kanamycin ($50 \mu\text{g ml}^{-1}$), if appropriate.

E. coli BL21(DE3)(pLysS)(pETS2-X-II) (Contzen & Stolz, 2000) was used for the synthesis of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate from protocatechuate and 4-sulfocatechol, respectively.

Plasmids and DNA manipulation techniques. Plasmid pBluescript II SK(+) was used for standard cloning experiments (Alting-Mees *et al.*, 1992). The plasmid vectors pJOE3075 and pAC28 were used for high levels of gene expression (Kholod & Mustelin, 2001; Stumpp *et al.*, 2000). The characteristics of all plasmids used are given in Table 1.

The genomic DNA of *H. intermedia* S1 was prepared after SDS lysis and phenol extraction as described by Eulberg *et al.* (1997). The genomic

DNA from *A. radiobacter* S2 was extracted using a DNeasy Tissue Kit (Qiagen) and the genomic DNA of *P. putida* using an E.Z.N.A Bacterial DNA Kit (Pheqlab Biotechnologie). Plasmid DNA from *E. coli* DH5 α was isolated with a GFX Micro Plasmid Prep kit (Pharmacia). Digestion of DNA with restriction endonucleases (MBI Fermentas), electrophoresis and ligation with T4 DNA ligase (MBI Fermentas) were performed according to standard procedures (Sambrook *et al.*, 1989). Transformation of *E. coli* was done by the method of Chung *et al.* (1989). For cloning of certain PCR products a T-vector was prepared as described by Marchuk *et al.* (1991).

PCR. Oligonucleotides were custom-synthesized (Eurogentec) according to known or deduced sequences from various CMLEs (Table 2). PCR mixtures ($50 \mu\text{l}$) for the amplification of genomic DNA contained 50 pmol each primer, $0.1\text{--}0.2 \mu\text{g}$ genomic DNA, 0.2 mM each deoxynucleotide triphosphate, *Taq* DNA polymerase ($2\text{--}5 \text{ U}$) and the corresponding reaction buffer (Eppendorf).

Table 2. Oligonucleotide primers used in this study

Position	Primer name	Amino acid sequence (if relevant)	Deduced primer sequence (5'→3')*
Downstream of <i>pcaG2</i> in <i>A. radiobacter</i> S2	S1-pcaG/se		CAAGCTACGTTGATCGTTACTGG
Consensus in CMLEs†	c-S1-se/c	EAALARA(Q/E)A	GAYGCNGCNCCTNGCNCGNGCNSAYGC
Consensus in CMLEs†	c-S1-as/a	MPHKRNPV	ATGCCWCARAAGAGYAARCCNGTD
Inverse PCR with genomic DNA of strain S1	pcaBS1_1930F		GTTGTCATCCACTGAGGTAGG
Inverse PCR with genomic DNA of strain S1	pcaBS1_1888R		GATTCCTAGCCAAGATCCGG
N terminus HiCMLE2	pcaB2S1_X_N		GCTGCCATATGAGTTTCTCTCCTCTTG
C terminus HiCMLE2	pcaB2S1_X_B		AACGGATCCTTTTAGGCCCGATGG
C terminus HiCMLE2 + His-tag	pcaB2S1-X_HisT		TTTTGGATCCGGCCCGATGGTTAAGGGC
N terminus ArCMLE2	CMLEII-X-N		ATAACATATGGCATTTCCTCCCTTGATTC
C terminus ArCMLE2	CMLEII-X-C		AAAAGGATCCTTGATCAGCGGGTAACATTTTCC
N terminus of CMLE from <i>P. putida</i> PRS2000	pPcaB-X-N2		AAAACATATGATGACCAACCAACTGTTTCG
C terminus of CMLE from <i>P. putida</i> PRS2000	pPcaB-X-C2		AAAAGGATCCTCACCCAGCACCGCACGCAGG

*The underlined sequences indicate the recognition sites for the restriction endonucleases *Nde*I and *Bam*HI.

†The oligonucleotides were deduced from the consensus sequences of different CMLEs. The amino acid sequences corresponded to aa 37–45 and 280–288 of ArCMLE2.

Partial inverse PCR. For the determination of the complete sequence of *pcaB2S1* from *H. intermedia* S1, a partial inverse PCR was performed (Pang & Knecht, 1997). The template was prepared by digesting chromosomal DNA (200 ng) of strain S1 for 1 h at 37 °C with *PauI* and religating the fragments obtained with T4 DNA ligase. The primers for this PCR (*pcaBS1_1930F* and *pcaBS1_1888R*; Table 2) were deduced from the fragment of *pcaB2S1* obtained previously. The following PCR program was used: an initial denaturation (95 °C, 1 min) was followed by 30 cycles consisting of an annealing step (60 °C, 1 min), a polymerization step (72 °C, 4 min) and a denaturation step (95 °C, 1 min). This resulted in the amplification of an approximately 2.4 kb fragment.

Construction of expression plasmids for the production of the CMLEs from *A. radiobacter* S2 (ArCMLE2), *H. intermedia* S1 (HiCMLE2) and *P. putida* PRS2000 (PpCMLE1) in *E. coli*.

The genes *pcaB2S1*, *pcaB2S2* and *pcaB* encoding HiCMLE2, ArCMLE2 and PpCMLE1, respectively, were amplified by PCR. The primers used simultaneously introduced *NdeI* sites upstream and *BamHI* sites downstream of the genes (see Table 2). The PCR products were then cleaved with *NdeI* and *BamHI* and inserted into *NdeI/BamHI*-cut expression vectors, resulting in amino-terminally His-tagged enzyme variants.

The *pcaB2S2* gene from *A. radiobacter* S2 was amplified by PCR using the oligonucleotide primers CMLEII-X-N and CMLEII-X-C. The following PCR program was used: an initial denaturation (94 °C, 1 min) was followed by 30 cycles consisting of an annealing step (65 °C, 1 min), a polymerization step (72 °C, 2 min) and a denaturation step (94 °C, 1 min). The amplified and digested DNA fragment was subsequently cloned into the expression vector pAC28 (Kholod & Mustelin, 2001).

HiCMLE2 was amplified using *Pfu* DNA polymerase (Stratagene) from the genomic DNA of *H. intermedia* S1 using primers *pcaB2S1_X-N* and *pcaB2S1_X-B* (Table 2), which were derived from the DNA sequence of the 2.4 kb DNA fragment obtained by inverse PCR (see above). The following PCR program was used: an initial denaturation (96 °C, 2 min) was followed by 30 cycles consisting of an annealing step (63.5 °C, 1 min), a polymerization step (72 °C, 2 min) and a denaturation step (96 °C, 30 s). The amplified product was cleaved with *NdeI* and *BamHI* and ligated into pJOE3075 (Stumpp *et al.*, 2000). Finally, *E. coli* JM 109 was transformed with the resulting plasmid pSBCMC2S1. A variant of the enzyme with an amino-terminal His-tag was constructed by cutting out *pcaB2S1* from pSBCMC2S1 using *NdeI* and *BamHI* and cloning it into the expression vector pAC28, giving plasmid pSHCMC2S1.

The *pcaB* gene was amplified using primers pPcaB-X-N2 and pPcaB-X-C2 (Table 2), which were deduced from the known nucleotide sequence of the gene [National Center for Biotechnology Information (NCBI) no. L17082]. The following PCR program was used: an initial denaturation (94 °C, 2 min) was followed by 30 cycles consisting of an annealing step (65 °C, 30 s), a polymerization step (72 °C, 1 min) and a denaturation step (94 °C, 30 s). The amplified product was cleaved with *NdeI* and *BamHI* and cloned into pAC28, giving pSHPPCMLE.

Expression of HiCMLE2, ArCMLE2 and PpCMLE1 in *E. coli*.

The three CMLEs were heterologously produced as amino-terminally His-tagged enzyme variants using *E. coli* BL21(DE3)(pLysS) Star(pSHCMC2S2) for ArCMLE2, *E. coli* BL21(DE3)(pLysS) Star(pSHCMC2S1) for HiCMLE2 and *E. coli* BL21(DE3)(pLysS) Star(pSHPPCMLE) for PpCMLE. The strains were grown in 300 ml LB medium (plus 50 µg kanamycin ml⁻¹) and the expression of the CMLEs was induced with 1 mM IPTG. The cells were harvested by centrifugation, resuspended in Tris/HCl buffer (50 mM, pH 8.0), disintegrated using a French Press, and cell extracts were prepared by centrifugation at 100 000 g for 30 min at 4 °C. The CMLEs were

purified using an Ni-NTA-column (25 ml; Qiagen) attached to an FPLC-apparatus (Pharmacia). The column was equilibrated with Tris/HCl (50 mM, pH 8.0), NaCl (300 mM), imidazole (20 mM) and DTT (1 mM). The CMLEs were eluted from the column by increasing the imidazole concentration to 200 mM in the buffer system described above. Fractions (5 ml each) were collected, the fractions with the CMLEs (usually fractions 3–5) were pooled and the elution buffer was removed using an ultrafiltration unit (20 ml Concentrator, 100 000 molecular mass cut-off; Vivascience). Finally, the concentrated enzyme solution was diluted with Tris/HCl (50 mM, pH 8.0). This procedure resulted in enzyme preparations of HiCMLE2 and ArCMLE2 which appeared on silver-stained gels to be greater than 99 % pure for both enzymes.

Nucleotide sequence analysis. The DNA sequences were determined by dideoxy-chain termination with double-stranded DNA of overlapping subclones in an automated DNA sequencing system (ALF-Sequencer; Amersham-Pharmacia) with fluorescently labelled primers.

Sequence analysis, database searches and comparisons were done with the Lasergene software package, version 5 (DNASTAR) and the BLAST search facility at NCBI (Altschul *et al.*, 1997). The alignments of the CMLEs were obtained with the program CLUSTALX using default parameters.

Estimation of protein concentration and enzyme assays. The protein content of cell-free extracts was determined by the method of Bradford (1976). Bovine serum albumin was used as standard. One unit of enzyme activity was defined as the amount of enzyme that converts 1 µmol substrate min⁻¹.

CMLE activity was measured using the spectrophotometric assay described by Ornston & Stanier (1966). The assays contained 100 µM 3-carboxy-*cis,cis*-muconate (synthesized enzymically from protocatechuate, see below) and 50 mM Tris/HCl buffer (pH 8.0) in a final volume of 1 ml. The decrease of absorption was determined at 260 nm. The reaction rates were calculated by using a molar extinction coefficient of $\epsilon_{260} = 7.3 \text{ mM}^{-1} \text{ cm}^{-1}$.

The turnover of 3-sulfomuconate was analysed by HPLC. The reaction mixtures contained 50 mM Tris/HCl buffer (pH 8.0) and 100 µM 3-sulfomuconate (synthesized enzymically from 4-sulfocatechol; see below). The reactions were usually monitored for 14 min, every 2 min aliquots (60 µl each) were removed, and the reactions were terminated in liquid nitrogen. The samples were separately thawed immediately before the HPLC analysis and the reaction rates were calculated from the time-dependent decrease in 3-sulfomuconate concentration.

Determination of molecular mass. The relative molecular masses of the native enzymes were determined by gel filtration using a Superdex 200 prep grade column (Amersham Biosciences) calibrated with an HMW Gel Filtration Calibration Kit (Amersham Biosciences). The subunit sizes were determined by SDS-PAGE with a Premixed Protein Molecular Mass Marker 14.4–97.4 kDa Kit (Roche) as reference proteins.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (1970) and the gels were routinely stained with Coomassie blue. In some experiments the gels were silver-stained using a Dodeca Silver Stain Kit (Bio-Rad).

HPLC. The turnover of 4-sulfocatechol and 3-sulfomuconate was analysed by reversed-phase HPLC (pump model 510 equipped with a photodiode array detector, model 996, and Millennium Chromatography Manager 2.0; Waters Associates). A reversed-phase column (250 × 4.0 mm i.d., packed with 3 µm particles of Nucleosil C18) was used. The mean flow rate was 1 ml min⁻¹. The separated compounds were detected photometrically at 210 nm

using a photodiode array detector. The solvent system consisted of 98.9% (v/v) water, 1% (v/v) methanol and 0.1% (v/v) H_3PO_4 . The mean retention times of 4-sulfocatechol, 3-sulfomuconate and 4-sulfomuconolactone under these chromatographic conditions were 3.7, 3.4 and 4.3 min, respectively.

Liquid chromatography (LC)-MS. Products were identified by using LC-MS (HP1100; Agilent) coupled to a triple quadrupole mass spectrometer (Quattro LC; Micromass) using electrospray ionization in the negative ion mode. Substrate solutions before and immediately after addition of enzyme and 20 min after addition of the enzyme were injected (20 μl) into the HPLC system without any pretreatment. Analytes were separated by ion-pair chromatography on a Luna C18(2) 3 μm column, 15 cm $\times$ 3 mm i.d. at 40 °C. Eluent A was $\text{H}_2\text{O}/\text{MeOH}$ (80:20, v/v) and eluent B, $\text{H}_2\text{O}/\text{MeOH}$ (5:95 v/v) with 5 mM tributylamine and 5 mM acetic acid. The gradient was 22% (v/v) B at 0 min, 22% B at 2 min, 90% B at 10 min, 90% B at 14 min and 22% B at 15 min, ready for injection after 22 min. A diode array detector and the mass spectrometer were coupled in series. The mass spectrometric interface was operated at a cone voltage of 18 V and a capillary voltage of 2.9 kV. The probe temperature was 220 °C and the source block temperature 120 °C. Product ion spectra were recorded at collision energies of 10 eV and 15 eV with a scan rate of 0.5 s.

Preparation of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate. The substituted muconates were prepared enzymically using protocatechuate dioxygenase activity (type 2), according to a method described by Ornston & Stanier (1966) for the preparation of 3-carboxy-*cis,cis*-muconate. The source of the required dioxygenase activity was *E. coli* BL21(DE3)(pLysS)(pETS2-X-II), which heterologously expresses P34OII from *A. radiobacter* S2 under the control of a phage T7 promoter (Contzen & Stolz, 2000). The strain was grown in 300 ml LB medium (plus 100 μg ampicillin ml^{-1}) and the expression of P34OII was induced with IPTG (0.4 mM). The cells were harvested by centrifugation, resuspended in Tris/HCl buffer (50 mM, pH 8.0), disintegrated and cell extracts prepared. The cell extracts (about 2 ml with a protein content of about 40 mg ml^{-1}) were mixed with 10 ml Tris/HCl (50 mM, pH 8.0) and protocatechuate or 4-sulfocatechol was added (4 mM each). The reaction mixtures were incubated at 30 °C on a laboratory shaker (100 r.p.m.).

The cell-free extract incubated with 4-sulfocatechol was analysed simultaneously by overlay spectra and HPLC analysis. The overlay spectra demonstrated the characteristic decrease in absorbance at 280 nm due to the turnover of 4-sulfocatechol (Hammer *et al.*, 1996). HPLC analysis with a reverse phase column [solvent system 98.9% (v/v) water, 1% (v/v) methanol, 0.1% (v/v) H_3PO_4] indicated that 4-sulfocatechol ($R_t = 3.7$ min; *in situ*-recorded $\lambda_{\text{max}} = 232$ nm, 281 nm) was converted to a new metabolite ($R_t = 3.5$ min; $\lambda_{\text{max}} = 205$ nm). The turnover of 4-sulfocatechol to 3-sulfomuconate was additionally verified by HPLC-MS/MS analysis. A new signal occurred ($R_t = 9.9$ min) with a molecular anion mass $[\text{M}-\text{H}]^-$ of m/z 221 that corresponds to sulfomuconate ($\text{C}_6\text{H}_6\text{O}_7\text{S}$). Fragment ions detected in product ion spectra indicated the presence of one sulfonate moiety and two carboxylate groups: m/z 177 ($\text{M}-\text{H}-\text{CO}_2$), 149 ($177-\text{CO}$), 139 ($\text{M}-\text{H}-\text{H}_2\text{SO}_3$), 113 ($177-\text{CO}_2$), 95 ($139-\text{CO}_2$) and 81 (HSO_3^-).

The solutions of the substituted muconates were separated from the proteins by ultrafiltration (30 000 molecular mass cut-off; Vivaspinn). The resulting filtrate containing 3-carboxy- or 3-sulfomuconate was used for the enzymic tests.

Chemicals. The chemicals used were obtained from Aldrich, Fluka, Merck and Sigma. 4-Sulfocatechol was synthesized according to the procedure described by Quilico (1927).

RESULTS

Identification, cloning and sequencing of the gene encoding the CMLE (type 2 enzyme) from *A. radiobacter* S2 (ArCMLE2)

The genes encoding P34OII from *A. radiobacter* S2 were previously cloned in the recombinant plasmid pMCS2-2 together with two ORFs which showed the highest degree of sequence similarity to a transport system for C4-carboxylates (TRAP-transporter). Approximately 3100 bp downstream of *pcaG2*, a third ORF was identified which showed sequence similarities to a putative regulator of the IclR family, and further downstream a part of a gene with sequence similarities to *pcaBs* was detected (Contzen & Stolz, 2000). In the course of the present study, the DNA insert on pMCS2-2 was completely sequenced and it was found that this gene, designated *pcaB2S2*, encodes ArCMLE2, a protein of 407 aa with 37–42% sequence identity to previously described CMLEs (Fig. 2).

Identification and cloning of the gene encoding HiCMLE2 from *H. intermedia* S1

The genes encoding the P34OII from *H. intermedia* S1 have already been cloned (Contzen *et al.*, 2001). Various attempts to obtain DNA fragments adjacent to *pcaH2G2* by partial inverse PCR only resulted in the cloning of a 1180 bp fragment which contained a putative transposase gene about 270 bp downstream of *pcaH2G2*. Therefore, sequence alignments were performed using the gene sequence obtained from strain S2 (see above) and those of the CMLEs from *Streptomyces* sp. strain 2065 (NCBI no. AAD40814), *Streptomyces coelicolor* (T35016), *Pseudomonas aeruginosa* PAO1 (NP_248921), *P. putida* (P32427), *Bradyrhizobium japonicum* USDA110 (O31385), *Acinetobacter* sp. ADP1 (Q59092) and *Agrobacterium tumefaciens* C58 (AAK88905). These alignments demonstrated the presence of two highly conserved regions among all sequences, and the sequences (encoding aa 37–45 and 280–288 in ArCMLE2) were used to design PCR primers (Table 2). A PCR using these primers and genomic DNA from strain S1 resulted in the amplification of a DNA fragment with the expected size (about 750 bp). The fragment was sequenced and shown to encode part of a putative CMLE. Because in *A. radiobacter* S2 *pcaH2G2* and *pcaB2S2* are physically connected, another PCR experiment was performed with genomic DNA from strain *H. intermedia* S1 using one of the primers deduced from the conserved regions in the CMLEs (c-S1-as/a; Table 2) and another one (S1-pcaG/se; Table 2) from the region downstream of *pcaG2* known from previous work. This PCR resulted in the amplification of an approximately 2 kb DNA fragment. Sequencing of this fragment demonstrated that the putative *pcaB2S1* was situated downstream of the putative transposase which, in turn, was located downstream of *pcaH2G2*. The missing part of *pcaB2S1* was finally obtained using partial inverse PCR (see Methods). The gene encoded HiCMLE2, a protein of 453 aa (HiCMLE2) with

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P. putida      : -----M-----TNQLFDAYFTAPAMREIFSDRGRLOGMLDFEAAARAEEAAG : 43
Ac. calcoaceticus : -----MSQLYASLFYQDRDTEIFSDRALVSYMVEAEVAAQAQQAQVG : 42
Ag. tumefaciens : MSLS-----PFEHPFLSGLFGDSEIVELFSARADIDAMIRFETAAQAQVG : 48
Ag. radiob. Type 2 : -----MA-----FSPLE-----DSLLGLPLFTTKEMRDALSERRFLALMLRVEAAARAQAAG : 48
H. interm. S1 type 2 : -----MS-----FSPLE-----DSEVLGNLFSTESMERVFSDSRYISFMFQVEGAAARAQAAG : 48

P. putida      : LVPMSAV---AAIEAACKAERYVDVGAANAIAATAGNSAIPLVKALGKVIASGVPEAEYVIL : 102
Ac. calcoaceticus : VIPSAAACDRCTCOQKTAIDKIDFADATATGLAGNIAIPFVKQLTAIVKDADEDAARYVEM : 104
Ag. tumefaciens : IISQDVLA---KATVSGLSSEFAADMTARHGVAKDGVVPELVQMRRAVAG---KAAEKVFP : 104
Ag. radiob. Type 2 : LVSGELA---NST---AGIDPGSLDIKVAEQTRIGAVPVIPFVKSQAHLX---PDVAGGVPF : 103
H. interm. S1 type 2 : LVPMSLA-A-TAI---ENVQREGLEPSTLARGSELGVPITPFVQAVQAKLP---PDLEPYFPF : 103

P. putida      : CARSODAMPGLVQLDRDALDLEADLGKLDATLSQALKHADTPMVGRWLODTPVTILGM : 164
Ac. calcoaceticus : CARSODILDEACILQCRDALAIVQVQCCYETALSQAATYRHQVMGRWLODLPITLGH : 166
Ag. tumefaciens : CARSODVLDPSLMRLKMATETIAARLGRLLDALGDIAARDGHNAITGYRMOATGITVAD : 166
Ag. radiob. Type 2 : CTSQDLMDDAILQMANAVDLIETDLRALLSSLCLEAEHCETPCIGRIACORASPVTGFP : 165
H. interm. S1 type 2 : SATQDILADTARVQLIRSDLPFLSHDLATVKNLASLAEKHRETPCVARIASQAPITFGY : 165

P. putida      : KLGVLGALTRHRQLQELGPPCWCSSGCAASGLAALGSKAM---PVAEALAEOLRLSLP : 222
Ac. calcoaceticus : KLRWASAFKRDLDRIATKARVLVAQLGCAVGSASLQDQGS---IVVEAYAKOLKKGQT : 224
Ag. tumefaciens : RAASWIAPLERHLLRLLETFAGSGFALQFGCAAGLEKLGDNAG---AVRADLAMRLGADK : 224
Ag. radiob. Type 2 : KVGWCVAIAEHLAQVEQLKRRVVLVSLAPVGLTQMGRAG---AVVTRTADLGHAVP : 223
H. interm. S1 type 2 : KVGWCVALSEHVEYLQTLRPHILVSLGCPVGLAALGDKGP---AVIDSFADLCHGSP : 223

P. putida      : EQPMHTQRDLRFEPASVGLVAGSLGRFGRDVSLLMQTEAGEVFPSPAPGKGGSSAMPKRN : 284
Ac. calcoaceticus : ACTWMBGRDRIVEIASVGLIITENVGRHARDVSLMTEIAEVFPPTAKRGSGSSAMPKRN : 286
Ag. tumefaciens : QQ-WQSGDGIAPFGNITSLVTEITLGRFGQDIALMAEIGT---GIRLGGGSGSSAMPKRN : 281
Ag. radiob. Type 2 : AMPWHTHRRISELVGLSWAILLGLAKMATDVHLATPEVGEVSPAVAGRGSGSSAMPKRN : 285
H. interm. S1 type 2 : PIWHTHRRISELVGLSWAILLGLAKMATDVHLATPEVGEVSPPEYPRGSGSSAMPKRN : 285

P. putida      : BVGAAVLIGAMTRVPLVSTLFAAMPQEHRSLSLGHAEHMTPEPICCLVSGALRQAVIAE : 346
Ac. calcoaceticus : BVAAASVLAANRPVPMSSIIYQSMVQEHRSLSLGHAEHMTPEIPQLTAGALERTLDVLK : 348
Ag. tumefaciens : BVNAETLAALARNFNAVQISALHQSLSVHEQERSGAGMLESLSQPMQVATG---ASLLIAE : 339
Ag. radiob. Type 2 : BVGSMILAQHSASVCHSLSTLSAMASLHERPVGAHHEHMTPEISLVGLAAGALREARLLAG : 347
H. interm. S1 type 2 : BVGSMILAAHGAAPGHVSTLMSLSASLHERPVGAHHEHMTPEISLVGLAAGALREARRVSG : 347

P. putida      : GIEVDAAARMRRNLDTQLGLVLAEAVSIVLARTFG----- : 380
Ac. calcoaceticus : GIEVNAENMHQNIETGLIMEAVMMALAPHMGRLENAHEVVEAACTAVAEQKHLKDIIISQ : 410
Ag. tumefaciens : RLAAQIDRLGTNGN----- : 353
Ag. radiob. Type 2 : GIEVDAAARMYRIELTNGMIFSDAVAGGLAQAMGRAEAYTAVEEEVANVVRSGGHFNVN--- : 406
H. interm. S1 type 2 : GIEVNVARMRRNLDTNGLFSDAAAVLSRSMGRKQAAHAAVEKAVSDVLARHQGSLLTCLAK : 409

P. putida      : -PRPCPLAGAMLP-----ASRGRTAAPACGAG----- : 407
Ac. calcoaceticus : VDEVQYFNPSQLDEIFKPSYLGNIQDIDAVLQEAKEGAK----- : 452
Ag. tumefaciens : ----- : -
Ag. radiob. Type 2 : ----- : -
H. interm. S1 type 2 : ---RHRNLAELRPAPDTTETSTRAAARTDAAIAHAKLISALNHRA : 453

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Fig. 2. Sequence alignment of different CMLEs. Residues that are identical in all sequences are highlighted by black boxes. The accession numbers and references for the published sequences of the enzymes from *P. putida*, *Acinetobacter* sp. ADP1 and *Agrobacterium tumefaciens* C58 are P32427 (Williams *et al.*, 1992), Q59092 (Kowalchuk *et al.*, 1994), and AAK88905 (Wood *et al.*, 2001), respectively.

significant homology to known CMLEs over its complete length (Fig. 2).

Comparison of the amino acid sequences of ArCMLE2 and HiCMLE2 with other CMLEs from different bacteria

A comparison of the deduced amino acid sequences of HiCMLE2 and ArCMLE2 and the sequences of other proven or putative CMLEs from the NCBI database clearly demonstrated that the two type 2 enzymes were more closely related to each other than to the other CMLEs (Fig. 3). The two presumed type 2 enzymes showed 50 % sequence identity, which was significantly higher than the sequence identities of 33–43 % observed between the two type 2 enzymes and all other previously described CMLEs (including all sequences of presumed CMLEs from various genome sequencing projects).

Comparison of the organization of the type 2 gene clusters to the protocatechuate gene clusters from different bacteria

The genes encoding the P34OII_s and CMLE2_s were organized in *H. intermedia* S1 and *A. radiobacter* S2 in a different way to all known variations in the structures of protocatechuate operons. Moreover, in strains S1 and S2 the genes encoding the P34OII_s and the CMLE2_s were separated by different genes (Fig. 4). This indicates that no strictly conserved 4-sulfocatechol degradation operon exists and

that the two pathways were not established in both strains by recent gene exchange events.

Expression of ArCMLE2 and HiCMLE2

The genes encoding HiCMLE2 and ArCMLE2 were cloned into the expression plasmid pAC28 under the control of the phage T7 promoter system (Kholod & Mustelin, 2001), giving plasmids pSHCMC2S1 and pSHCMC2S2, respectively. This resulted in the formation of carboxy- and amino-terminally His-tagged proteins. The expression of the genes by the addition of IPTG resulted in the formation of additional peptides in the crude extracts. The sizes of the additional bands were estimated by SDS-PAGE for both HiCMLE2 and ArCMLE2 at approximately 42 kDa. The CMLE activities of the recombinant *E. coli* strains were tested using the spectrophotometric enzyme assay described by Ornston & Stanier (1966). Thus it was shown that the cell extracts from *E. coli* BL21(DE3)(pLysS)(pSHCMC2S1) and *E. coli* BL21(DE3)(pLysS)(pSHCMC2S2) converted 3-carboxy-*cis,cis*-muconate with specific activities of 0.67 and 0.5 U (mg protein)⁻¹, respectively.

Turnover of 3-sulfomuconate by HiCMLE2 and ArCMLE2

Cell extracts were prepared from *E. coli* BL21(DE3)(pLysS) Star carrying plasmids pSHCMC2S1 or pSHCMC2S2 and incubated with 3-sulfomuconate (1–4 mM). These reactions were analysed by HPLC because 3-sulfomuconate, unlike most other substituted muconates, does not have pronounced

Fig. 3. Dendrogram showing the phylogenetic relatedness of CMLEs from different bacterial sources. The dendrogram was produced using the programs CLUSTALX and TREEVIEW (Page, 1996). The references for the sequences from *P. putida*, *Acinetobacter calcoaceticus* and *Agrobacterium tumefaciens* are given in the legend to Fig. 2. The accession numbers and references for the sequences from *B. japonicum*, *Rhodococcus opacus* 1CP, *P. putida* DOT-T1E, *P. aeruginosa*, *Streptomyces* sp. 2065 and *S. coelicolor* are O31385 (Lorite *et al.*, 1998), AAC38245 (Eulberg *et al.*, 1998), AAD39559 (Ramos *et al.*, 1998), NP 248921 (Stover *et al.*, 2000), AAD40814 (Iwagami *et al.*, 2000) and T35016, respectively.

absorbance at 260 nm (Feigel & Knackmuss, 1993). HPLC analysis with a reverse phase column (using the same solvent system as above) demonstrated that 3-sulfomuconate ($R_t=3.5$ min; $\lambda_{\max}=205$ nm) was converted to a new metabolite ($R_t=4.2$ min; $\lambda_{\max}=215$ nm). The subsequent analysis of the reaction by HPLC-MS/MS confirmed the turnover of 3-sulfomuconate to 4-carboxymethylene-4-sulfo-but-2-en-olide (4-sulfomuconolactone). A new signal was

detected ($R_t = 9.0$ min) with an m/z of 221 for the molecular anion $[M-H]^-$. Less fragmentation occurred than for sulfomuconate, as the cyclic lactone system remained intact and only the exocyclic functional groups were split off as fragments: 177 ($M-H-CO_2$), 139 ($M-H-H_2SO_3$), 95 ($139-CO_2$) and 81 (HSO_3^-). No reactions were observed in control experiments without added cell extracts or with cell extracts from *E. coli* which did not express the recombinant enzymes.

Fig. 4. Structures of gene clusters for the catabolism of protocatechuate and 4-sulfocatechol from *H. intermedia* S1 and *A. radiobacter* S2 in comparison to the protocatechuate gene clusters from *R. opacus*, *Acinetobacter calcoaceticus*, *P. putida* and *Agrobacterium tumefaciens* (Eulberg *et al.*, 1998; Parke, 1995).

The recombinant CMLEs were purified by affinity chromatography using their His-tags. The purified enzymes showed (determined with 3-carboxy-*cis,cis*-muconate as substrate) pH optima in Tris/HCl buffer at pH 8.0 and about 50 % of their maximal activity in Tris/HCl, pH 9.0, or histidine/HCl, pH 5.8. The enzymes showed significantly reduced activities in sodium/potassium phosphate/citrate buffers compared to histidine/HCl buffers at the same pH, as found previously for the CMLE from *P. putida* (Ornston, 1966).

In the spectrophotometric tests with 3-carboxy-*cis,cis*-muconate, $v_{\max}$ values of 1.5 and 1.3 U (mg protein)⁻¹ were determined for HiCMLE2 and ArCMLE2, respectively (Table 3). These values were significantly lower than those described for the CMLE from *P. putida* [547 U (mg protein)⁻¹] (Ornston, 1966). Furthermore, HiCMLE2 and ArCMLE2 also showed slightly higher K_m values for 3-carboxy-*cis,cis*-muconate than the enzyme from *P. putida* (Table 3).

Finally, different concentrations (0.5–4 mM) of 3-sulfomuconate were incubated with the purified enzymes, five aliquots of each were taken after different time intervals (2–10 min) and the turnover of 3-sulfomuconate was analysed by HPLC. These experiments suggested that both enzymes showed higher $v_{\max}$ values and lower K_m values with the sulfonated substrate compared to the carboxylated muconate (Table 3).

Turnover of 3-sulfomuconate by PpCMLE from *P. putida* PRS2000

We tested if also the 'archetypal' CMLE from the protocatechuate pathway of *P. putida* PRS2000 (PpCMLE1) could convert 3-sulfomuconate. Cell extracts were prepared from cells of *P. putida* PRS2000 grown with protocatechuate (to induce the formation of PpCMLE1). The cell extracts converted 3-carboxy-*cis,cis*-muconate and surprisingly also 3-sulfomuconate with specific activities of 2.0 and 0.13 U (mg protein)⁻¹, respectively. To confirm the ability of PpCMLE1 to convert 3-sulfomuconate, the gene was amplified from the chromosomal DNA of *P. putida*

PRS2000 and cloned into the expression vector pAC28 (see Methods). The enzyme was expressed as a His-tagged enzyme variant and partially purified on an Ni-NTA column to a specific activity of 240 U mg⁻¹ with 3-carboxy-*cis,cis*-muconate as substrate [compared to a $v_{\max}$ reported for the purified enzyme of 457 U (mg protein)⁻¹ by Ornston (1966)]. The same enzyme preparation was incubated with 3-sulfomuconate (0.5, 1, 2 and 3 mM) in the HPLC assay used for the CMLEs. Thus it was found that PpCMLE indeed converted 3-sulfomuconate. In these tests the highest reaction rates were observed with 0.5 mM 3-sulfomuconate [8.5 U (mg protein)⁻¹] and a pronounced decrease in the reaction rates was observed using higher substrate concentrations.

DISCUSSION

The results of the present study and our previous investigations of the 4-sulfocatechol-oxidizing P34OII demonstrated that the degradation of 4-sulfocatechol to 4-sulfomuconolactone is catalysed by two enzymes which are clearly derived from the protocatechuate branch of the β -ketoadipate pathway. This became evident from the range of substrates converted by both groups of enzymes and the sequence data obtained. It was found previously that the P34OII from *H. intermedia* S1 and *A. radiobacter* S2 converted 4-sulfocatechol and protocatechuate (Contzen *et al.*, 2001). In accordance with this observation, the present study demonstrated that ArCMLE2 and HiCMLE2 converted carboxylated and sulfonated muconates. Furthermore, it was previously shown by multiple sequence alignments that both subunits of the P34OII from strains S1 and S2 clustered together with the corresponding subunits of the protocatechuate 3,4-dioxygenases from various protocatechuate-degrading organisms (Contzen & Stolz, 2000; Contzen *et al.*, 2001) and here we show that the two type 2 CMLEs are also homologues of the CMLEs from protocatechuate-degrading organisms.

The sequence comparisons demonstrated that the two 3-sulfomuconate-converting CMLEs investigated in this study

Table 3. Kinetic data for the turnover of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate by the 3-carboxy-*cis,cis*-muconate cycloisomerases from *H. intermedia* S1 and *A. radiobacter* S2

MM, molecular mass; 3 CM, 3-carboxy-*cis,cis*-muconate; 3SM, 3-sulfomuconate. The data were calculated by non-linear regression using Prism 4 software (GraphPad Software).

Enzyme	Source	MM per subunit (kDa)	Substrate	K_m (mM)	$v_{\max}$ (U mg ⁻¹)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	k_{cat}/K_m 3SM:3CM
HiCMLE2	<i>H. intermedia</i> S1	48.4	3CM	0.15 ± 0.04	1.5 ± 0.31	73	486	0.74
			3SM	7.1 ± 1.9	53 ± 10	2565	362	
ArCMLE2	<i>A. radiobacter</i> S2	42.5	3CM	0.15 ± 0.07	1.3 ± 0.4	55	368	0.22
			3SM	15.3 ± 5.7	29.5 ± 9.2	1254	82	
PpCMLE1*	<i>P. putida</i>	42.4	3CM	0.075	547	23 200	309 200	

*Data taken from Ornston (1966).

are more closely related to each other than to the corresponding enzymes from the protocatechuate pathways of various other bacteria. This was especially evident for ArCMLE2 from *A. radiobacter* S2 which is more closely related to the isofunctional HiCMLE2 from the taxonomically distantly related *Hydrogenophaga* strain (these organisms belong to the *Alpha*- or *Betaproteobacteria*) than to the previously described CMLEs from the protocatechuate pathways of other agrobacteria (Parke, 1996; Wood *et al.*, 2001). This clearly suggested a horizontal transfer of the gene encoding ArCMLE2 from a different genetic background to strain S2.

Unfortunately, only very limited information is available about the enzymic characteristics of bacterial CMLEs. This is mainly due to the commercial unavailability and instability of the enzyme substrate 3-carboxy-*cis,cis*-muconate. Thus, since the pioneering work of Ornston (1966), who described the first purification and characterization of a CMLE (from *P. putida*), no other bacterial CMLEs have been studied in any detail with regard to their enzymic characteristics.

It was a surprising observation that PpCMLE1, which belongs to the protocatechuate pathway of *P. putida*, was also able to convert 3-sulfomuconate. This suggested that for CMLEs, no strict separation into type 1 enzymes (which only accept carboxylated substrates) and type 2 enzymes (which convert carboxylated plus sulfonated substrates) can be used as previously suggested for protocatechuate-cleaving activities (Feigel & Knackmuss, 1993). Nevertheless, it is evident that there are huge differences in the specific activities of the different types of CMLEs for the turnover of 3-carboxy-*cis,cis*-muconate. The comparison of the data given by Ornston (1966) for the CMLE from *P. putida* (and also our own results obtained with the His-tagged enzyme variant PpCMLE1) with HiCMLE2 and ArCMLE2, which participate in the degradation of 4-sulfocatechol, showed that the CMLE from *P. putida* converted 3-carboxy-*cis,cis*-muconate with a much higher $v_{\max}$ and a slightly lower K_m than the type 2 enzymes from *H. intermedia* S1 and *A. radiobacter* S2. The CMLE from *P. putida* therefore seems to be much better adapted to the turnover of 3-carboxy-*cis,cis*-muconate than the type 2 enzymes studied here.

The analysis of the turnover of 3-sulfomuconate by PpCMLE did not allow a reliable calculation of the kinetic constants because of the observed substrate inhibition kinetics and the inherent difficulties of the HPLC test applied. Nevertheless, it became evident that the relative activities of this enzyme with 3-carboxy-*cis,cis*-muconate in comparison to the turnover of 3-sulfomuconate are much higher than the corresponding values for HiCMLE2 and ArCMLE2. Surprisingly, it appears that the specific activities of the purified PpCMLE with 3-sulfomuconate are higher than those determined for HiCMLE2 and ArCMLE2. This indicates that, for the turnover of 3-sulfomuconate from the point of enzyme specificity and activity, no specifically adapted CMLEs are necessary. To analyse this apparent

contradiction more accurately, we are currently studying the turnover of 3-sulfomuconate by other CMLEs originating from the protocatechuate pathways of different bacteria.

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REFERENCES

- Alexander, M. & Lustigman, B. K. (1966). Effect of chemical structure on microbial degradation of substituted benzenes. *J Agric Food Chem* **14**, 410–413.
- Alonso, M. C., Castillo, M. & Barceló, D. (1999). Solid-phase extraction procedure of polar benzene- and naphthalenesulfonates in industrial effluents followed by unequivocal determination with ion-pair chromatography/electrospray-mass spectrometry. *Anal Chem* **71**, 2586–2593.
- Alting-Mees, M. A., Sorge, J. A. & Short, J. M. (1992). pBluescript II: multifunctional cloning and mapping vectors. *Methods Enzymol* **216**, 483–495.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**, 248–254.
- Brilon, C., Beckmann, W. & Knackmuss, H.-J. (1981). Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl Environ Microbiol* **42**, 44–55.
- Chung, C. T., Niemela, S. L. & Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci U S A* **86**, 2172–2175.
- Contzen, M. & Stolz, A. (2000). Characterization of the genes for two protocatechuate 3,4-dioxygenases from the catechol-4-sulfonate degrading bacterium *Agrobacterium radiobacter* strain S2. *J Bacteriol* **182**, 6123–6129.
- Contzen, M., Wittich, R.-M., Knackmuss, H.-J. & Stolz, A. (1996). Degradation of benzene-1,3-disulfonate by a mixed bacterial culture. *FEMS Microbiol Lett* **136**, 45–50.
- Contzen, M., Moore, E. R. B., Blümel, S., Stolz, A. & Kämpfer, P. (2000). *Hydrogenophaga intermedia* sp. nov., a 4-aminobenzenesulfonate degrading organism. *Syst Appl Microbiol* **23**, 487–493.
- Contzen, M., Bürger, S. & Stolz, A. (2001). Cloning of the genes for a 4-sulfocatechol-oxidizing protocatechuate 3,4-dioxygenase from *Hydrogenophaga intermedia* S1 and identification of the amino acid residues responsible for the ability to convert 4-sulfocatechol. *Mol Microbiol* **41**, 199–205.
- Cook, A. M., Laue, H. & Junker, F. (1999). Microbial desulfonation. *FEMS Microbiol Rev* **22**, 399–419.
- Dong, W., Eichhorn, P., Radajewski, S., Schleheck, D., Denger, K., Knepper, T. P., Murrell, J. C. & Cook, A. M. (2004). *Parvibaculum lavamentivorans* converts linear alkylbenzenesulfonate surfactant to sulfophenylcarboxylates, α,β -unsaturated sulfophenylcarboxylates

and sulfophenyldicarboxylates, which are degraded in communities. *J Appl Microbiol* **96**, 630–640.

Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H.-J. (1974). Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. *Arch Microbiol* **99**, 61–70.

Eulberg, D., Golovleva, L. A. & Schlömann, M. (1997). Characterization of catechol catabolic genes from *Rhodococcus opacus* 1CP. *J Bacteriol* **179**, 370–381.

Eulberg, D., Lakner, S., Golovleva, L. A. & Schlömann, M. (1998). Characterization of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP: evidence for a merged enzyme with 4-carboxymuconolactone-decarboxylating and 3-oxoadipate enol-lactone-hydrolyzing activity. *J Bacteriol* **180**, 1072–1081.

Feigel, B. J. & Knackmuss, H.-J. (1988). Bacterial catabolism of sulfanilic acid via catechol-4-sulfonic acid. *FEMS Microbiol Lett* **55**, 113–118.

Feigel, B. J. & Knackmuss, H.-J. (1993). Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two-species bacterial culture. *Arch Microbiol* **159**, 124–130.

Hammer, A., Stolz, A. & Knackmuss, H.-J. (1996). Purification and characterization of a novel type of protocatechuate-3,4-dioxygenase with the ability to oxidize 4-sulfocatechol. *Arch Microbiol* **166**, 92–100.

Hughes, E. J., Shapiro, M. K., Houghton, J. E. & Ornston, L. N. (1988). Cloning and expression of *pca* genes from *Pseudomonas putida* in *Escherichia coli*. *J Gen Microbiol* **134**, 2877–2887.

Iwagami, S. G., Yang, K. & Davies, J. (2000). Characterization of the protocatechuate catabolic gene cluster from *Streptomyces* sp. strain 2065. *Appl Environ Microbiol* **66**, 1499–1508.

Kholod, N. & Mustelin, T. (2001). Novel vectors for co-expression of two proteins in *E. coli*. *Biotechniques* **31**, 322–328.

Knepper, T. P. (2002). Mass spectrometric strategies for the analysis of polar industrial chemicals and their by-products in wastewater and surface water. *J Chromatogr A* **974**, 111–121.

Kowalchuk, G. A., Hartnett, G. B., Benson, A., Houghton, J. E., Ngai, K. L. & Ornston, L. N. (1994). Contrasting patterns of evolutionary divergence within the *Acinetobacter calcoaceticus* *pca* operon. *Gene* **146**, 23–30.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lorite, M. J., Sanjuan, J., Velasco, L., Olivares, J. & Bedmar, E. J. (1998). Characterization of *Bradyrhizobium japonicum* *pcaBDC* genes involved in 4-hydroxybenzoate degradation. *Biochim Biophys Acta* **1397**, 257–261.

Marchuk, D., Drumm, M., Saulino, A. & Collins, F. S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res* **19**, 1154.

Nörtemann, B., Baumgarten, J., Rast, H. G. & Knackmuss, H.-J. (1986). Bacterial communities degrading amino- and hydroxy-naphthalene-2-sulfonates. *Appl Environ Microbiol* **52**, 1195–1202.

Ohe, T., Ohmoto, T., Kobayashi, Y., Sato, A. & Watanabe, Y. (1990). Metabolism of naphthalenesulfonic acids by *Pseudomonas* sp. TA-2. *Agric Biol Chem* **54**, 669–675.

Ornston, L. N. (1966). The turnover of catechol and protocatechuate to β -ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. *J Biol Chem* **241**, 3787–3794.

Ornston, L. N. & Stanier, R. Y. (1966). The turnover of catechol and protocatechuate to β -ketoadipate by *Pseudomonas putida*. I. Biochemistry. *J Biol Chem* **241**, 3776–3786.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Pang, K. M. & Knecht, D. A. (1997). Partial inverse PCR: a technique for cloning flanking sequences. *Biotechniques* **22**, 1046–1048.

Parke, D. (1995). Supraoperonic clustering of *pca* genes for catabolism of the phenolic compound protocatechuate in *Agrobacterium tumefaciens*. *J Bacteriol* **177**, 3808–3817.

Parke, D. (1996). Characterization of PcaQ, a LysR-type transcriptional activator required for catabolism of phenolic compounds from *Agrobacterium tumefaciens*. *J Bacteriol* **178**, 266–272.

Quilico, A. (1927). Azione dell'acido amminosolfonico sui difenoli. *Gazz Chim Ital* **57**, 793–802.

Ramos, J. L., Duque, E., Godoy, P. & Segura, A. (1998). Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J Bacteriol* **180**, 3323–3329.

Riediker, S., Suter, M. J.-F. & Giger, W. (2000). Benzene- and naphthalenesulfonates in leachates and plumes of landfills. *Water Res* **34**, 2069–2079.

Ruckstuhl, S., Suter, M. J.-F., Kohler, H.-P. E. & Giger, W. (2002). Leaching and primary biodegradation of sulfonated naphthalenes and their formaldehyde condensates from concrete superplasticizers in groundwater affected by tunnel construction. *Environ Sci Technol* **36**, 3284–3289.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Schleheck, D., Knepper, T. P., Fischer, K. & Cook, A. M. (2004). Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria. *Appl Environ Microbiol* **70**, 4053–4063.

Schulz, S., Dong, W., Groth, U. & Cook, A. M. (2000). Enantiomeric degradation of 2-(4-sulfophenyl)butyrate via 4-sulfocatechol in *Delftia acidovorans* SPB1. *Appl Environ Microbiol* **66**, 1905–1910.

Stover, C. K., Pham, X.-Q. T., Erwin, A. L. & 28 other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**, 959–964.

Stumpp, T., Wilms, B. & Altenbuchner, J. (2000). Ein neues, L-Rhamnose-induzierbares Expressionssystem für *Escherichia coli*. *Biospektrum* **6**, 33–36.

Thurnheer, T., Zürcher, D., Höglinger, O., Leisinger, T. & Cook, A. M. (1990). Initial steps in the degradation of benzene sulfonic acid, 4-toluene sulfonic acids, and orthonilic acid in *Alcaligenes* sp. strain O-1. *Biodegradation* **1**, 55–64.

Tully, P. S. (1997). Sulfonic acids. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th edn, vol. 23, pp. 194–217. New York: Wiley.

Wellens, H. (1990). Zur biologischen Abbaubarkeit mono- und disubstituierter Benzolderivate. *Z Wasser Abwasser Forsch* **23**, 85–98.

Williams, S. E., Woolridge, E. M., Ransom, S. C., Landro, J. A., Babbitt, P. C. & Kozarich, J. W. (1992). 3-Carboxy-*cis,cis*-muconate lactonizing enzyme from *Pseudomonas putida* is homologous to the class II fumarase family. A new reaction in the evolution of a mechanistic motif. *Biochemistry* **31**, 9768–9776.

Wittich, R.-M., Rast, H. G. & Knackmuss, H.-J. (1988). Degradation of naphthalene-2,6- and naphthalene-1,6-disulfonic acid by a *Moraxella* sp. *Appl Environ Microbiol* **54**, 1842–1847.

Wood, D. W., Setubal, J. C., Kaul, R. & 47 other authors (2001). The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**, 2317–2323.

Yang, J., Wang, Y., Woolridge, E. M., Arora, V., Petsko, G. A., Kozarich, J. W. & Ringe, D. (2004). Crystal structure of 3-carboxy-*cis,cis*-muconate lactonizing enzyme from *Pseudomonas putida*, a fumarase class II type cycloisomerase: enzyme evolution in parallel pathways. *Biochemistry* **43**, 10424–10434.