Molekularbiologische Untersuchungen des Abbauweges von 4-Sulfocatechol durch *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2

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Die experimentellen Arbeiten für die vorliegende Dissertation wurden unter der Leitung von Prof. Dr. Andreas Stolz am Institut für Mikrobiologie der Universität Stuttgart durchgeführt. Hiermit erkläre ich, dass die Arbeiten für die vorliegende Dissertation von mir selbständig durchgeführt wurden.

Einige Ergebnisse der vorliegenden Arbeit wurden bereits auf Tagungen vorgestellt (VAAM-Tagung in Braunschweig 2004, Jena 2006)

Inhaltsverzeichnis

Abkürzungen

4ABS	Sulfanilat (4-Aminobenzolsulfonat)
ArCMLE1	3-Carboxymuconat-Cycloisomerase Typ I aus *A. radiobacter*
ArCMLE2	3-Carboxymuconat-Cycloisomerase Typ II aus *A. radiobacter*
ArSLH	4-Sulfolacton-Hydrolase aus *A. radiobacter*
BDS	1,3-Benzoldisulfonat
CHM	2-Hydroxy-4-carboxymuconat
4CL	4-Carboxymuconolacton
CMLE	3-Carboxymuconat-Cycloisomerase
3CM	3-Carboxy-*cis,cis*-muconat
Da, kDa	Dalton, Kilodalton
DNA	Desoxyribonucleinsäure
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EL	β- Ketoadipatenollacton
FPLC	Fast Protein Liquid Chromatography
HiCMLE2	3-Carboxymuconat-Cycloisomerase Typ II aus *H. intermedia*
HiSLH	4-Sulfolacton-Hydrolase aus *H. intermedia*
HPLC/MS/MS	Massenspektrometrie
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-1-thio-β-D-galaktosid
KA	β-Ketoadipat
K_m	Michaelis- Konstante
λ	Wellenlänge
LAS	lineare Alkylbenzolsulfonate
LB	Luria-Bertani-Medium
NCBI	National Center for Biotechnology Information
NMR	Kernresonanzspektroskopie

MA	Maleylacetat
OD	optische Dichte
P34O	Protocatechuat-3,4-Dioxygenase
PAGE	Polyacrylamidgelelektrophorese
PDC	2-Pyron-4,6-dicarboxylat
PDCH	2-Pyron-4,6-dicarboxylat-Hydrolase
PC	Protocatechuat
PCR	Polymerase-Kettenreaktion
SDS	Natriumdodecylsulfat
4SC	4-Sulfocatechol
SLH	4-Sulfolacton-Hydrolase
4SL	4-Sulfolacton
3SM	3-Sulfomuconat
SPB	2-(4'-Sulfophenyl) butyrat
Tris	Tris-(hydroxymethyl)-aminomethan
%(v/v)	Volumenprozent
%(w/v)	Gewichtsprozent

Abstract

A bacterial two-species culture consisting of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 was studied. This mixed culture was able to use the xenobiotic compound 4-aminobenzenesulfonate (4ABS) as the sole source of carbon, nitrogen, sulfur and energy. In the mixed culture strain S1 oxidizes 4ABS to 4-sulfocatechol (4SC), which was identified as the central metabolite in the bacterial degradation of other substituted benzenesulfonates. 4SC is excreted by strain S1 into the medium and taken up by strain S2. Both strains degrade 4SC through a metabolic pathway which is analogous to the protocatechuate branch of the β-ketoadipate pathway. Initially, 4SC is oxidized by an *ortho*-cleavage mechanism to 3-sulfomuconate (3SM). Subsequently, the further metabolism of 3-sulfomuconate is catalysed by certain 3-carboxy-*cis,cis*-muconate lactonizing enzymes (CMLE) to 4-carboxymethylen-4-sulfobut-2-en-olid (4-sulfomuconolactone, 4SL). The desulfonation of 4SL to maleylacetate (MA) is catalyzed by 4-sulfomuconolactone hydrolases. The intermediately formed maleylacetate is reduced to β-ketoadipate by maleylacetate reductases.

It was previously shown that the 4SC oxidizing enzymes from strains S1 and S2 also oxidized protocatechuate and that the 3SM lactonizing activities also converted 3-carboxy-*cis,cis*-muconate (3CM). Therefore, the enzymes converting only protocatechuate and its ensuing products were called type I enzymes. The enzymes which also converted the sulfonated structural analogues were described as type II enzymes.

In the course of the present study, the other enzymes which are involved in the 4-sulfocatechol degradative pathway were characterized. Initially, the conversion of 3SM to 4SL was analyzed in comparison to the transformation of 3CM to 4-carboxymuconolactone (4CL).The genes *pcaB1* and *pcaB2* from the strains S1 and S2 were expressed in *E. coli* and the encoded CMLEs of type I and type II were purified and characterized. It was shown that the CMLEs of type II from *H. intermedia* and *A. radiobacter* (HiCMLE2 and ArCMLE2) and also the CMLE of type I from *A. radiobacter* (ArCMLE1) converted 3SM to 4SL. The kinetic parameters of the three enzymes were determined with 3CM and 3SM as substrates. The HiCMLE2 and ArCMLE2 converted 3SM with higher specific activities than 3CM (V_{max}= 53 and 29.5 U/mg with 3SM compared to 1.5 and 1.3 U/mg with 3CM). Surprisingly, ArCMLE1 had a higher specific activity with 3SM as substrate than the other CMLEs of typ II (V_{max}= 130 U/mg with 3SM compared to 2270 U/mg with 3CM).

The genes that coded for the 4-sulfomuconolactone hydrolases (SLH) were identified in both strains downstream of the *pcaB2* genes. The genes were expressed in *E. coli* and the 4-sulfomuconolactone hydrolases were purified and characterized. The SLH from *H. intermedia* (HiSLH) converted 4SL with higher V_{max}- and K_m-values than the enzyme from *A. radiobacter* (ArSLH). However, the catalytic constants of both enzymes were very similar (k_{cat}/K_m=1417 mM^{-1} min^{-1} for HiSLH compared to 1233 mM^{-1} min^{-1} for ArSLH). The enzymatic conversion of 4-sulfomuconolactone resulted in the release of approximately equimolar amounts of maleylacetate and sulfite. 4SL showed a structural resemblance to 2-pyrone-4,6-dicarboxylate (PDC), which was identified as an intermediate in an extradiol degradative pathway of protocatechuate via a protocatechuate 4,5-dioxygenase reaction. Significant sequence similarities between the SLHs from strains S1 and S2 and the 2-pyrone-4,6-dicarboxylate hydrolase (PDCH) from *Sphingomonas paucimobilis* SYK-6 were found. The incubation of HiSLH with PDC and the incubation of PDCH with 4SL demonstrated no cross-reactivities for both enzymes. Therefore, it was suggested that 4-sulfomuconolactone hydrolases form an independent group of enzymes. Further, sequence comparisons demonstrated some sequence similarities between the carboxyterminal parts of SLHs and the cyclic amidases to which enzymes such as D-hydantoinases and dihydropyrimidases belong which contain divalent cations in their active site (e.g. Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+}). Therefore, the metal content of the HiSLH was determined using plasma mass spectrometry (ICP-MS). Thus, Zn^{2+}-ions were identified in the active center of HiSLH.

Finally, the proposed degradative pathway of 4SC was confirmed with the recombinant enzymes using *in-situ* ^{1}H-NMR. Thus, the structure of each intermediate of the degradative pathway was proven.

Kurzfassung

Eine bakterielle Zweispezies-Kultur aus den Stämmen *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2 wurde untersucht. Diese Mischkultur war in der Lage, den naturfremden Stoff 4-Aminobenzolsulfonat (4ABS) als einzige Kohlenstoff-, Stickstoff-, Schwefel- und Energiequelle zu nutzen. In der Mischkultur oxidiert der Stamm S1 das 4ABS zu 4-Sulfocatechol (4SC), welches als zentraler Metabolit im bakteriellen Abbau von anderen substituierten Benzolsulfonaten identifiziert wurde. 4SC wird zum Teil in das Medium ausgeschieden und von dem Stamm S2 aufgenommen. Das 4SC wird von den beiden Stämmen über einen dem Protocatechuat-Zweig des β-Ketoadipat-Weges analogen Stoffwechselweg abgebaut. Hierbei wird 4SC von den beiden Stämmen durch eine intradiole Ringspaltung zu 3-Sulfomuconat (3SM) umgesetzt. 3-Sulfomuconat wird anschließend zu 4-Carboxymethylen-4-sulfobut-2-en-olid (4-Sulfolacton, 4SL) durch 3-Carboxymuconat-Cycloisomerasen (CMLE) cycloisomerisiert. Die Desulfonierung des 4-Sulfolactons zu Maleylacetat (MA) erfolgt durch 4-Sulfolacton-Hydrolasen (SLH). Maleylacetat wird daraufhin von den Stämmen S1 und S2 mit Hilfe von Maleylacetat-Reduktasen zu β-Ketoadipat reduziert.

Es wurde in früheren Untersuchungen gezeigt, dass die 4SC oxidierenden Enzyme aus den Stämmen S1 und S2 auch Protocatechuat und die 3SM umsetzenden Aktivitäten auch 3-Carboxy-*cis,cis*-muconat (3CM) als Substrate akzeptierten. Aus diesem Grund wurden die Enzyme, die nur Protocatechuat und dessen Folgeprodukte umsetzen, als Typ I Enzyme bezeichnet. Dagegen wurden die Enzyme, die auch die sulfonierten Struktur-Analoga umsetzen, als Typ II Enzyme angesprochen.

Im Rahmen der vorliegenden Arbeit wurden die weiteren am 4-Sulfocatechol Abbauweg beteiligten Enzyme charakterisiert. Hierbei wurde zunächst der Umsatz von 3SM zu 4SL im Vergleich mit dem Umsatz von 3CM zu 4-Carboxymuconolacton (4CL) untersucht. Hierzu wurden die Gene *pcaB1* und *pcaB2* aus den Stämmen S1 und S2 in *E. coli* überexprimiert. Die kodierten CMLEs Typ I und Typ II wurden aufgereinigt und charakterisiert. Es wurde gezeigt, dass sowohl die CMLEs vom Typ II aus *H. intermedia* und *A. radiobacter* (HiCMLE2 und ArCMLE2) als auch die CMLE von Typ I aus *A. radiobacter* (ArCMLE1) 3SM zu 4SL umsetzten. Die grundlegenden kinetischen Parameter der drei Enzyme wurden mit 3CM und 3SM als Substrate bestimmt. Obwohl die HiCMLE2 und ArCMLE2 eine gewisse Spezialisierung an den Umsatz von 3SM zeigten (V_{max} = 53 bzw. 29,5 U/mg mit 3SM im Vergleich zu 1,5 bzw. 1,3 U/mg mit 3CM), besaß die ArCMLE1 eine höhere

spezifische Aktivität mit 3SM als Substrat (V_{max} = 130 U/mg mit 3SM im Vergleich zu 2270 U/mg mit 3CM).

Die Gene, die für die 4-Sulfolacton-Hydrolasen kodierten, konnten „downstream“ der *pcaB2*-Gene in den beiden Stämmen identifiziert werden. Die Gene wurden in *E. coli* exprimiert und die 4-Sulfolacton-Hydrolasen aufgereinigt und charakterisiert. Die SLH aus *H. intermedia* (HiSLH) setzte 4SL mit höheren V_{max}- und K_m-Werten als das Enzym aus *A. radiobacter* (ArSLH) um, allerdings waren die katalytischen Konstanten der beiden Enzyme sehr ähnlich (k_{cat}/K_m = 1417 mM^{-1} min^{-1} für HiSLH im Vergleich zu 1233 mM^{-1} min^{-1} für ArSLH). Beim Umsatz von 4-Sulfolacton wurde die Freisetzung annähernd äquimolarer Mengen von Maleylacetat und Sulfit nachgewiesen.

4-Sulfolacton (4SL) zeigte eine Strukturähnlichkeit zu 2-Pyron-4,6-dicarboxylat (PDC), einem Intermediat in einem extradiolen Abbauweg von Protocatechuat über eine Protocatechuat-4,5-Dioxygenase Reaktion. Es wurden signifikante Sequenzähnlichkeiten zwischen den 4-Sulfolacton-Hydrolasen aus den Stämmen S1 und S2 und der 2-Pyron-4,6-dicarboxylat-Hydrolase (PDCH) aus dem Stamm *S. paucimobilis* nachgewiesen. Umsatzversuche mit 4SL und PDC zeigten, dass HiSLH und ArSLH eine eigenständige Gruppe von Enzymen darstellen, da die SLHs kein PDC und die PDHC kein 4SL umsetzten. Weiterführende Sequenzvergleiche zeigten Sequenzähnlichkeiten zwischen den carboxyterminalen Bereichen der 4-Sulfolacton-Hydrolasen und den zyklischen Amidasen, zu denen Enzyme wie z.B. D-Hydantoinasen und Dihydropyrimidasen gehören, die Mn^{2+}-, Mg^{2+}-, Zn^{2+}-, Ni^{2+}- oder Co^{2+}- Ionen in ihren aktiven Zentren beinhalten. Deshalb wurde der Metallgehalt der HiSLH mittels Massenspektrometrie mit induktiv gekoppeltem Plasma (ICP-MS) bestimmt, wobei Zn^{2+}-Ionen im aktiven Zentrum der HiSLH identifiziert wurden.

Abschließend wurde der Abbauweg von 4SC durch die rekombinanten Enzyme mittels *in-situ* ^{1}H-Kernresonanzspektroskopie bestätigt und die Entstehung jedes Reaktionsprodukts im Verlauf des Abbauweges nachgewiesen.

1. Einleitung

1.1. Aromatische Sulfonsäuren als Fremd- und Schadstoff in der Natur

Die Verknüpfung von Biosynthese und Biodegradation ist ein Grundprinzip der Natur. Die biologischen Prozesse dafür, sowohl die Synthese der Naturstoffe als auch ihre Mineralisierung, konnten sich über große Zeiträume entwickeln. Allerdings wurden mit dem Anbruch des technischen Zeitalters zum Teil beträchtliche Mengen naturfremder Stoffe (Xenobiotika) in die Umwelt eingebracht. Viele dieser Verbindungen weisen chemische Strukturprinzipien auf, die nicht in der Natur vorkommen. Deshalb unterliegen viele Xenobiotika oft nur einem stark verlangsamten Abbau in der Biosphäre.

Zu den Umweltschadstoffen gehören chlorierte, nitrierte und sulfonierte Aromaten, da diese Substanzen vielfach durch Mikroorganismen nur unvollständig oder verlangsamt abgebaut werden. Aromatische Sulfonsäuren besitzen eine große Bedeutung für die chemische Industrie. Die Sulfonsäurefunktion lässt sich synthetisch einfach in organische Verbindungen einführen und erhöht die Löslichkeit der Produkte gegenüber den nicht sulfonierten Edukten drastisch. Aromatische Sulfonsäuren finden insbesondere Verwendung als Detergenzien, Dispergiermittel, Farbstoffe, optische Aufheller, Ionenaustauscher und Pharmazeutika.

Im Gegensatz zu ihrer großtechnischen Herstellung in der chemischen Industrie werden die aromatischen Sulfonsäuren in der Natur höchstens randständig gebildet. Es überrascht daher nicht, dass eine Reihe von Substanzen aus dieser Verbindungsklasse in der Umwelt über längere Zeiträume persistieren. Schwefelhaltige organische Verbindungen bilden in den Abwässern vieler industrieller und kommunaler Kläranlagen einen hohen Prozentsatz der trinkwasserrelevanten Verbindungen, die nicht oder schlecht biologisch abbaubar sind und nicht oder nur unvollständig an Aktivkohlefilter binden (Völker & Sontheimer, 1984).

Die mengenmäßig bedeutendste Gruppe an industriell produzierten aromatischen Sulfonsäuren stellen die linearen Alkylbenzolsulfonsäuren (LAS) dar, die in den meisten Textilwaschmitteln als anionische Tenside eingesetzt werden. Die Weltjahresproduktion an LAS wurde auf 1,8 Millionen Tonnen geschätzt (Berth & Jeschke, 1989). Die im Handel befindlichen LAS sind Mischungen verschieden langer unverzweigter Alkylverbindungen, die jeweils einen 4′-Sulfophenylrest tragen. Für die LAS wurde in verschiedenen Modellsystemen gezeigt, dass ein quantitativ bedeutender Anteil nur einem Primärabbau unterliegt und aus den LAS gebildete sulfonierte Metabolite am Ablauf der Kläranlagen nachgewiesen werden können (Schöberl, 1989; Schöberl & Kunkel 1977). Die linearen

Alkylbenzolsulfonsäuren sind verhältnismäßig toxisch für aquatische Lebensformen, wobei die Wirkung von LAS auf Wasserlebewesen primär auf die Schädigung der dünnen und empfindlichen Membranen in den Kiemen zurückgeführt wird (Malle, 1993). Fische, die längere Zeit in LAS-haltigem Wasser lebten, reicherten die LAS oder aus diesen gebildete Metabolite in ihrem Gewebe gegenüber der Umgebungskonzentration an (Anliker *et al*., 1981; Kimerle *et al*., 1981; Wakabayashi *et al*., 1978; Tolls *et al*., 1994). Ein Partialabbau der LAS, der zu einer Zerstörung der oberflächenaktiven Eigenschaften dieser Verbindung führt, erniedrigt die akute Toxizität stark. So besitzt z.B. 4´-Sulfophenylundecanoat für *Daphnia magna* eine LC_{50} von 200 mg/ml und das Abbauprodukt 4´-Sulfophenylbutyrat eine LC_{50} von 5000-10000 mg/l (Kimerle & Swisher, 1977).

1.2. Der bakterielle Abbau von sulfonierten Aromaten

Aufgrund der vielfach beobachteten Schaumbildung wurden in den frühen sechziger Jahren des 20. Jahrhunderts die in vielen Waschmitteln verwendeten verzweigtkettigen Alkylbenzolsulfonate, die in der Umwelt persistieren, durch die linearen Alkylbenzol-sulfonate (LAS) ersetzt, da sich diese als biologisch abbaubar erwiesen hatten (Cain, 1991). Trotzdem betrug der Anteil der sulfonierten aromatischen Verbindungen 1978 zwischen 7 und 15 % der organischen Gesamtlast des Rheins (Malle, 1978).

Die Frage nach dem Verbleib sulfonierter aromatischer Verbindungen in der Natur weckte verhältnismäßig früh das wissenschaftliche Interesse. Im Rahmen von Untersuchungen zum Abbau der sulfonierten Detergenzien wurden die ersten Arylsulfonate-abbauenden Bakterienstämme isoliert. So wurden von Cain und Farr (1968) Pseudomonaden angereichert, die in der Lage waren, Benzosulfonat und 4-Methylbenzolsulfonat zu mineralisieren. In späteren Arbeiten wurden weitere Organismen und Mischkulturen mit 2-, 3,- und 4-Aminobenzolsulfonat, 3-Nitrobenzolsulfonat, 4-Hydroxybenzolsulfonat, 4-Methylbenzol-sulfonat, 4-Sulfobenzoat, 4-Sulfophthalat und auch mit verschiedenen substituierten Naphthalinsulfonaten als Kohlenstoff- und Energiequelle angereichert. Allerdings wurden die Abbauwege dieser Verbindungen nur in wenigen Fällen aufgeklärt (Thurnheer *et al*., 1986, 1988; Locher *et al*., 1989; Richter & Wittich, 1994; Brilon *et al*., 1981a,b; Nörtemann *et al*., 1986; Ohe & Watanabe, 1986; Wittich *et al*., 1988; Rozgaj & Glancer-Soljan, 1992; Tan *et al*., 2005).

1.3. Der mikrobielle Abbau von Sulfanilat

Sulfanilat (4-Aminobenzolsulfonat = 4ABS) ist ein gutes Beispiel für die vielfältigen Verwendungsmöglichkeiten sulfonierter aromatischer Verbindungen: 4ABS wurde hauptsächlich in der UdSSR und der VR China als Fungizid eingesetzt bzw. als Konservierungsmittel verwendet (Feigel, 1990; Hopper, 1994). Sulfanilat dient auch als Zwischenprodukt bei der industriellen Synthese verschiedener Verbindungen. Desweiteren wurde Sulfanilat als Intermediat des mikrobiellen Abbaus von Pflanzenschutzmitteln (wie z.B. Asulam) nachgewiesen (Babiker & Duncan, 1977; Smith & Walker, 1977; Franci *et al*., 1981).

Ein mikrobieller Abbau von Sulfanilat durch Bakterienmischkulturen aus Belebtschlamm wurde von Orshanskaya *et al*. (1975) beschrieben. Die Mischpopulation war in der Lage, 4ABS als einzige Kohlenstoff-, Stickstoff- und Energiequelle vollständig zu verwerten. Dagegen konnte aber keine der aus dieser Mischkultur isolierten Reinkulturen Sulfanilat vollständig mineralisieren. Auch Wellens (1990) wies die biologische Abbaubarkeit von 4ABS durch eine nicht definierte Mischpopulation nach. Ein vollständiger Abbau von Sulfanilat durch Reinkulturen wurde von Thurnheer *et al.* (1986) beschrieben. Nach Anreicherung mit 4ABS wurden die Isolate S1 und S3 erhalten, die mit Sulfanilat als einziger Kohlenstoff- und Energiequelle wuchsen. Allerdings wurden die Isolate nicht weiter untersucht. In einer späteren Untersuchung einer anderen Arbeitsgruppe wurde der Stamm *Pseudomonas paucimobilis* isoliert, der in der Lage war, 4-Aminobenzolsulfonat abzubauen (Perei *et al*., 2001). Auch dieser Stamm konnte mit 4ABS als einziger Kohlenstoff- und Stickstoffquelle wachsen.

Feigel isolierte bereits 1985 eine 4ABS abbauende Bakterienkultur, die in der Lage war, 4ABS als Kohlenstoff-, Stickstoff-, Schwefel- und Energiequelle zu nutzen. Für den Abbau war eine bakterielle Zweispezies-Kultur verantwortlich, die aus den Stämmen *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2 bestand (Feigel, 1985; Contzen *et al*., 2000). Der Metabolismus von Sulfanilat verlief hierbei zu einem großen Teil in Analogie zum Protocatechuat-Zweig des β-Ketoadipat-Weges (Abb.1). Die postulierte initiale oxygenolytische Desaminierung des 4ABS zu 4-Sulfocatechol (4SC) kann hierbei nur vom Stamm S1 katalysiert werden. Das vom Stamm S1 gebildete 4SC wird zum Teil in das Medium ausgeschieden und vom Stamm S2 aufgenommen. 4SC wird von den beiden Stämmen durch eine intradiole Ringspaltung zu 3-Sulfomuconat (3SM) umgesetzt. 3-

Sulfomuconat wird anschließend zu 4-Carboxymethyl-4-sulfobut-2-en-olid (4-Sulfolacton 4SL)cycloisomerisiert.

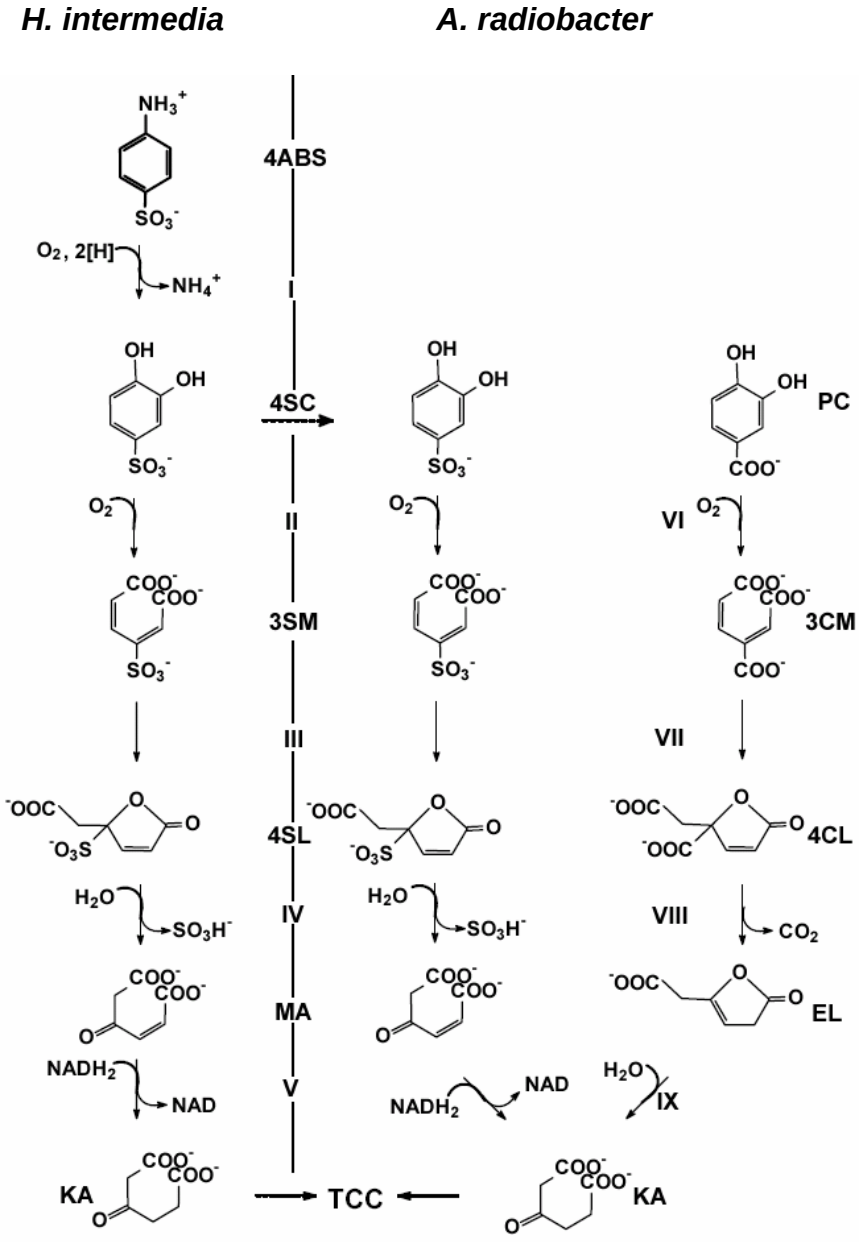

Abb. 1: Abbau von 4-Aminobenzolsulfonat über 4-Sulfocatechol durch eine syntrophe Mischkultur aus den Stämmen *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2 im Vergleich zu dem Abbau von Protocatechuat durch den Stamm S2 (Feigel & Knackmuss, 1993).

4ABS: 4-Aminobenzolsulfonat (Sulfanilat); 4SC: 4-Sulfocatechol; 3SM: 3-Sulfomuconat; 4SL: 4-Sulfolacton, MA: Maleylacetat; KA: β-Ketoadipat; PC: Protocatechuat; 3CM: 3-Carboxymuconat; 4CL: Carboxymuconolacton; EL: β-Ketoadipatenollacton; TCC: Tricarbonsäurezyklus

I: 4-Aminobenzolsulfonat-3,4-Dioxygenase; II: Protocatechuat-3,4-Dioxygenase Typ II; III: 3-Carboxymuconat-Cycloisomerase Typ II; IV: 4-Sulfolacton-Hydrolase; V: Maleylacetat-Reduktase; VI : Protocatechuat-3,4-Dioxygenase Typ I; VII: 3-Carboxymuconat-Cycloisomerase Typ I ; VIII: 4-Carboxymuconolacton- Decarboxylase; IX: β-Ketoadipatenollacton-Hydrolase

Für die Desulfonierung des 4-Sulfolactons zu Maleylacetat (MA) wurde ein hydrolytischer Mechanismus vorgeschlagen (Feigel & Knackmuss, 1993). Maleylacetat wird daraufhin von den Stämmen S1 und S2 mit Hilfe von Maleylacetat-Reduktasen zu β-Ketoadipat reduziert. An diesem Punkt konvergiert der 4ABS-Abbauweg mit dem β-Ketoadipat-Weg, über den viele aromatische Naturstoffe mineralisiert werden (Ornston & Stanier, 1966; Ornston, 1966; Parke *et al.*, 2000).

Der Abbau von 4ABS zeigt deutliche Parallelen zum Abbau des Naturstoffes Protocatechuat (PC) über den β-Ketoadipat-Weg. So kommt es beim Abbau von Protocatechuat zunächst zu einer intradiolen Spaltung des Protocatechuats zu 3-Carboxy-*cis,cis*-muconat (3CM), während bei der Ringspaltung des 4-Sulfocatechol 3-Sulfomuconat entsteht. 3-Carboxy-*cis,cis*-muconat wird im nächsten Schritt zu 4-Carboxymuconolacton (4CL) cycloisomerisiert. Die analoge Reaktion im 4-Sulfocatechol-Abbau stellt die Lactonisierung des 3SM zu 4SL dar. Allerdings entspricht die anschließende Decarboxylierung des 4CL zu β-Ketoadipatenollacton nicht der Desulfonierung des 4-Sulfolactons, da die Carboxylgruppe des 4CL nicht wie die Sulfonatgruppe des 4SL anionisch eliminiert werden kann.

Feigel und Knackmuss (1993) zeigten, dass die 4SC oxidierenden Enzyme aus den Stämmen S1 und S2 auch Protocatechuat und die 3SM umsetzenden Aktivitäten auch 3CM als Substrate akzeptierten. Sie vermuteten, dass es sich bei den beiden diese Reaktionsschritte katalysierenden Enzyme um spezifisch adaptierte Formen von Protocatechuat-3,4-Dioxygenasen bzw. 3-Carboxymuconat-Cycloisomerasen aus dem Protocatechuat-Zweig des β-Ketoadipat-Weges handelte. Sie zeigten weiterhin, dass im Stamm S2 auch ein „konventioneller" Protocatechuat-Abbauweg induziert wurde, dessen Enzyme die sulfonierten Analoga nicht als Substrate akzeptierten. Aus diesem Grund wurden die Enzyme, die nur Protocatechuat und dessen Folgeprodukte umsetzen, als Typ I Enzyme bezeichnet. Dagegen wurden die Enzyme, die auch die sulfonierten Struktur-Analoga umsetzen als Typ II Enzyme angesprochen. Nach dieser Definition handelt es sich bei der 4-Sulfocatechol spaltenden Aktivität um eine Protocatechuat-3,4-Dioxygenase Typ II (P34O-II) und bei der 3-Sulfomuconat lactonisierenden Aktivität um eine 3-Carboxymuconat-Cycloisomerase Typ II (CMC-II). In späteren Untersuchungen konnte zudem gezeigt werden, dass die P34O-IIs aus den Stämmen S1 und S2 verschiedene charakteristische Eigenschaften der P34O-Is aufweisen. So waren auch die P34O-IIs aus zwei verschiedenen Untereinheiten aufgebaut und besaßen ebenfalls Fe^{3+}-Ionen im katalytischen Zentrum. Auch die Molekulargewichte der α-und β-Untereinheiten in den P34O-IIs entsprachen weitgehend den für andere P34Os beschriebenen Größen. Desweiteren belegte die Sequenzierung der

aminoterminalen Bereiche der Untereinheiten der P34O-IIs aus den Stämmen S1 und S2, dass die P34O-IIs mit den Protocatechuat-3,4-Dioxygenasen aus dem β-Ketoadipat-Weg evolutionär verwandt sind (Hammer, 1995; Hammer *et al.*, 1996).

1.4. 4-Sulfocatechol als zentraler Metabolit im Abbau substituierter Benzolsulfonate

In späteren Arbeiten wurde 4SC als zentraler Metabolit im bakteriellen Abbau von anderen substituierten Benzolsulfonaten identifiziert. Offensichtlich werden nicht nur viele substituierte Benzolsulfonsäuren, wie z.B. 4-Aminobenzolsulfonsäure, 4-Phenolsulfonsäure und disulfonierte Benzolsulfonsäuren, sondern auch die in großen Mengen in Kläranlagen eingeleiteten linearen Alkylbenzolsulfonsäuren (LAS) über 4-Sulfocatechol abgebaut (Cook *et al*. 1999; Abb. 2).

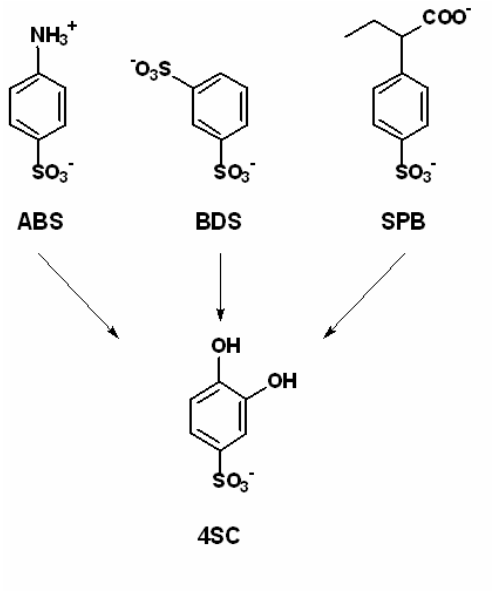

Abb.2: 4-Sulfocatechol als zentraler Metabolit im Abbau substituierter Benzolsulfonate. ABS 4-Aminobenzolsulfonat (Sulfanilat) (Feigel & Knackmuss, 1993); BDS 1,3-Benzoldisulfonat (Contzen *et al*., 1996); SPB 2-(4'-Sulfophenyl) butyrat (Cook *et al*., 1999)

1.5. Genetische Untersuchungen zum Abbauweg des 4-Sulfocatechols

Der β-Ketoadipat-Weg ist sowohl biochemisch als auch genetisch intensiv untersucht worden. Die Enzyme des Protocatechuat-Zweiges des β-Ketoadipat-Weges werden durch die *pca*-Gene kodiert. Zu den Enzymen und Genen des Protocatechuat-Abbauweges und über die Organisation und Regulation der *pca*-Gene liegen relativ viele Informationen vor (Ornston & Stanier, 1966; Ornston, 1966; Zylstra *et al*., 1989; Hartnett *et al*., 1990; Hartnett & Ornston, 1994; Harwood *et al*., 1994; Parke, 1995). Im Gegensatz hierzu wurde der Abbauweg

sulfonierter Benzole molekularbiologisch bis heute kaum untersucht. Die ersten genetischen Untersuchungen auf diesem Gebiet wurden mit der Mischkultur aus den Stämmen S1 und S2 durchgeführt (Contzen & Stolz, 2000; Contzen *et al.*, 2001). Hierbei wurde ein ca. 13 kb umfassendes Gencluster für den Abbau von Protocatechuat (*pca*-Gene vom Typ I) aus dem Stamm S2 kloniert und sequenziert. Sequenzvergleiche zeigten, dass es sich bei diesem Cluster um das typische *pca*-Gencluster eines *Agrobacterium*-Stammes handelt. Die Gene *pcaH1G1*, die für die Protocatechuat-3,4-Dioxygenase Typ I kodieren, wurden anschließend in *E. coli* exprimiert. Die P34O-I konnte Protocatechuat oxidieren aber nicht 4-Sulfocatechol. Weiterhin wurde aus dem Stamm S2 noch ein ca. 11 kb großes DNA-Fragment kloniert und sequenziert. Das für die P34O-II kodierende Gen wurde auf diesem DNA-Fragment identifiziert. Das Expressionsprodukt dieses Gens konnte sowohl 4SC als auch PC umsetzen. Ungefähr 4 kb „downstream" von *pcaH2G2* konnte ein Gen identifiziert werden, das signifikante Sequenzähnlichkeiten zu den *pcaB*-Genen aufwies und als *pcaB2* angesprochen wurde (Contzen *et al.*, 2001). Im Rahmen dieser Untersuchungen konnte gezeigt werden, dass *pcaH2G2* und *pcaB2* phylogenetisch mit den klassischen *pca*-Genen verwandt sind. Zwischen *pcaG2* und *pcaB2* wurden weitere „Open Reading Frames" identifiziert, die für zwei Transportproteine und einen Regulator kodieren könnten. Auf dem sequenzierten DNA-Fragment mit *pcaH2G2* und *pcaB2* wurden „downstream" von *pcaB2* weitere Gene identifiziert, die als Gene der 4-Sulfolacton-Hydrolase bzw. einer Maleylacetat-Reduktase angesprochen wurden.

Aus *H. intermedia* S1 konnten von Contzen *et al.* (2001) *pcaH2* und ein Großteil von *pcaG2* kloniert und sequenziert werden. Auch für diesen Stamm wurde gezeigt, dass *pcaH2* phylogenetisch mit klassischen *pca*-Genen verwandt ist. Über Sequenzvergleiche wurden Aminosäurereste identifiziert, in denen die β-Untereinheiten der beiden P34O-IIs vom Konsensus der bisher bekannten P34Os von Typ I abwichen. Eine ortsspezifische Mutagenese in diesem identifizierten Bereich resultierte in einer mutierten Form der P34O-I des Stammes S2, die in der Lage war, 4SC zu oxidieren (Contzen *et al.* 2001).

Im Rahmen der vorliegenden Arbeit sollten zunächst die Gene, die für die 3-Carboxymuconat-Cycloisomerase Typ II, die 4-Sulfolacton-Hydrolase und die Maleylacetat-Reduktase kodieren, aus dem Stamm S1 isoliert und sequenziert werden. Im Folgenden sollten die an dem Abbauweg von 4-Sulfocatechol beteiligten Enzyme charakterisiert werden. Hierzu sollten die Gene für die 3-Carboxymuconat-Cycloisomerasen Typ I und Typ II, und die Gene der 4-Sulfolacton-Hydrolasen aus den Stämmen S1 bzw. S2 in

Expressionsvektoren kloniert und die rekombinanten Enzyme aufgereinigt werden. Das Ziel der Arbeit sollte es sein, die molekulare Evolution der 4-Sulfocatechol abbauende Enzyme zu untersuchen und Unterschiede gegenüber Protocatechuat abbauenden Enzymen zu identifizieren.

2. Ergebnisse

2.1. Synthese von 4-Sulfocatechol

Da 4-Sulfocatechol (4SC) das Ausgangssubstrat dieser Untersuchung und kommerziell nicht erhältlich war, wurde es nach der Vorschrift von Quilico (1927) hergestellt. Hierzu wurde in einem erhitzten Dreihalskolben bei ca. 170°C Brenzkatechin (25 g) geschmolzen und unter ständigem Rühren fein pulverisierte Sulfaminsäure (9,7 g) in kleinen Portionen zugegeben. Nach der Zugabe von ca. 2/3 der angegebenen Menge an Amidosulfonsäure veränderte sich die Konsistenz der Reaktionsmischung und es bildete sich eine bräunliche Masse. Nachdem die restliche Amidosulfonsäure zugegeben worden war, wurde noch 30 min bei obiger Temperatur gerührt. Die abgekühlte, feste Masse wurde in 20 ml dest. Wasser gelöst. Um überschüssiges Brenzkatechin zu entfernen, wurde das Produkt mehrmals mit Diethylether extrahiert. Nach der Abtrennung der organischen Phase wurde die Wasserphase lyophilisiert. Reine Kristalle des Sulfobrenzkatechins konnten durch Umkristallisierung aus Wasser nicht gewonnen werden. Mittels ^{1}H-NMR-, ^{13}C-NMR- und HPLC-Analysen wurde 4-Sulfocatechol als Produkt dieser Präparation nachgewiesen (Feigel, 1990). Der Anteil von 4-Sulfocatechol in dem Pulver wurde mit ca. 95 % abgeschätzt. Neben 4-Sulfocatechol wurde in dem Pulver 3-Sulfocatechol (ca. 5 %) mittels Massenspektrometrie nachgewiesen. Das so erhaltene Pulver wurde für die weiteren Analysen eingesetzt.

2.2. Enzymatische Herstellung von 3-Carboxy-*cis*,*cis*-muconat und 3-Sulfomuconat

Ein wichtiges Ziel der hier beschriebenen Untersuchungen bestand in vergleichenden enzymatischen Untersuchungen zum Umsatz von 3-Carboxy- und 3-Sulfomuconat durch die verschiedenen Typen der 3-Carboxymuconat-Cycloisomerasen. Da beide Substrate kommerziell nicht erhältlich sind und zumindest für das 3-Sulfomuconat bis heute keine chemische Synthese beschrieben worden ist, mussten beide Substrate für die geplanten enzymatischen Untersuchungen biologisch präpariert werden. Hierzu wurde ein rekombinanter *E. coli* Stamm eingesetzt, der die Protocatechuat-3,4-Dioxygenase Typ II aus dem Stamm S2 rekombinant exprimiert (Contzen *et al.*, 2001).

4-Sulfocatechol bzw. Protocatechuat (4 mM, 1 ml in Tris/HCl pH 8) wurde mit einem Rohextrakt (100 µl, 45 mg/ml) aus dem Stamm *E.coli* BL21(DE3)(plysS)(pETS2-X-II) inkubiert, in dem die Protocatechuat-3,4-Dioxygenase Typ II vorhanden war. Die Reaktion wurde bei 30°C unter Schütteln bei 1400 rpm durchgeführt und mittels HPLC analysiert (s. Material und Methoden, Manuskript 1) bis 4-Sulfocatechol bzw. Protocatechuat zu 3-Sulfomuconat bzw. 3-Carboxymuconat vollständig umgesetzt wurden.
Die 3-Sulfomuconat- bzw. 3-Carboxymuconat-Lösungen wurden mit Hilfe einer Ultrafiltrationseinheit („VIVASPIN 2ml Concentrator", Membrane 10,000 MWCO PES, VIVASCIENCE, Sartorius Group) von den Proteinen abgetrennt und für die weiteren Versuche eingesetzt.

2.3. Klonierung und Expression der *pcaB*-Gene aus *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2

Die am Abbau von Protocatechuat beteiligten „Typ I Gene" waren in *A. radiobacter* S2 offenbar in einem typischen „*Agrobacterium*-Protocatechuat-Operon" organisiert, wie es zuvor auch für einen Protocatechuat abbauenden Stamm von *A. tumefaciens* beschrieben worden war (Parke *et al.,* 1997). In dem Gen-Cluster von *A. radiobacter* S2 konnte direkt „down-stream" der für die Protocatechuat-3,4-Dioxygenase (Typ I) kodierenden Gene (*pcaH1G1*) ein Gen identifiziert werden, das signifikante Sequenzhomologien zu bekannten 3-Carboxymuconat-Cycloisomerasen (PcaB) aufwies und daher als *pcaB1S2* angesprochen wurde (Contzen & Stolz, 2000). Das Gen wurde anschließend sequenziert (Basta, 2000). Für eine rekombinante Expression wurde *pcaB1S2* in den Expressionsvektor pJOE3075 (Stumpp *et al.,* 2000) kloniert. Hierzu wurde das Gen *pcaB1S2* mit Hilfe der PCR mit den Oligonukleotiden CMLEI-X-N und CMLEI-His-C (s.Material und Methoden, Manuskript 2) ohne Stoppsignal amplifiziert. Das amplifizierte DNA-Fragment wurde mit den Endonucleasen *Nde*I und *Bam*HI verdaut und zwischen die *Nde*I- und *Bam*HI-Schnittstellen in pJOE3075 ligiert. Durch diese Klonierungsstrategie wurde die 3-Carboxymuconat-Cycloisomerase Typ I aus *A. radiobacter* S2 (ArCMLE1) mit sechs Histidin-Resten („His-Tag") an dem carboxyterminalen Ende fusioniert. Das daraus resultierende Plasmid (pSHCMC1S2) wurde zur Transformation von *E. coli* JM109 verwendet. Die Expression der ArCMLE1 wurde nach der Zugabe von 0,2 % (w/v) L-Rhamnose zu der Kultur (OD_{546nm}= 0,2-0,3) in LB/Ampicillin Medium induziert. Anschließend wurde ein Zellextrakt aus *E. coli* JM 109(pSHCMC1S2) in Tris/HCl (50 mM, pH 8) hergestellt. Die Expression des Enzyms wurde mittels SDS-PAGE analysiert und die Größe der neuen Proteinbande wurde mit ca. 37

kDa abgeschätzt. Die Aktivität der rekombinanten ArCMLE1 in dem Rohextrakt wurde nach der Vorschrift von Ornston & Stanier (1966) spektrophotometrisch mit 3-Carboxy-*cis,cis*-muconat (3CM) bestimmt. Die photometrische Analyse zeigte (Abb.3), dass der Zellextrakt von *E. coli* JM109(pSHCMC1S2) 3-Carboxy-*cis,cis*-muconat (3CM) mit einer spezifischen Aktivität von 9,4 U/mg zu 4-Carboxymuconolacton (4CL) umsetzte (s. Manuskript 2).

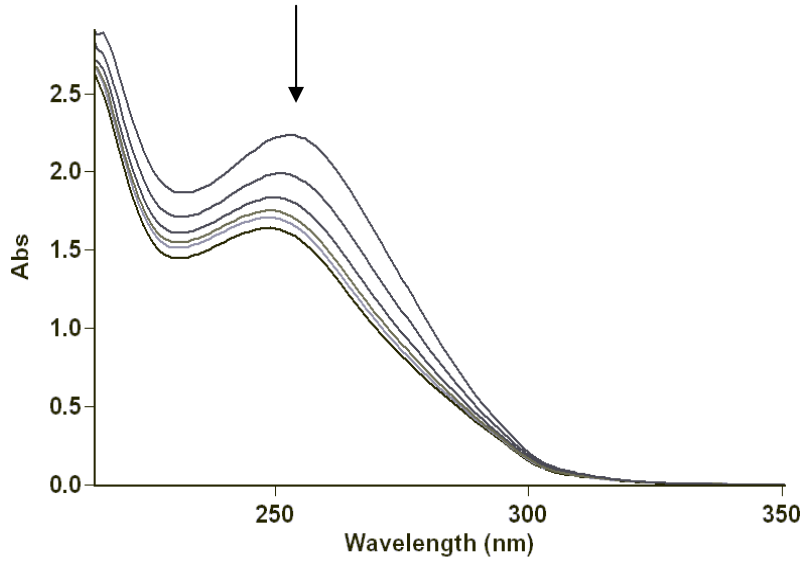

Abb. 3: Umsatz von 3-Carboxy-*cis,cis*-muconat (3CM) durch einen Rohextrakt von *E. coli* JM109 (pSHCMC1S2). 3CM (0,1 mM) wurde mit 1µl Zellextrakt (4,5 mg/ml) in 1ml Tris/HCl (50 mM, pH 8) bei Raumtemperatur für 3 min inkubiert. Es wurden 3 Spektren pro Minute aufgenommen.

Bereits in den vorangegangenen Untersuchungen konnte gezeigt werden, dass die für die Typ II-Enzyme in *A. radiobacter* S2 kodierenden Gene offenbar eine völlig andere Organisation als die entsprechenden Gene des klassischen Protocatechuat-Abbauwegs aufweisen (Contzen & Stolz, 2000). Hierbei wurde etwa 4,3 kb „down-stream" von *pcaH2G2* ein weiteres putatives Operon identifiziert, das drei Gene enthielt, die Sequenzhomologien zu 3-Carboxymuconat-Cycloisomerasen, verschiedenen Hydrolasen und Maleylacetat-Reduktasen aufwiesen. Es war daher wahrscheinlich, dass das für die putative 3-Carboxymuconat-Cycloisomerase kodierende Gen für das gesuchte „TypII" Enzym kodierte. Dieses Gen aus *A. radiobacter* (*pcaB2S2*) wurde sequenziert (Basta, 2000). Die abgeleitete Aminosäuresequenz von *pcaB2S2* zeigte 32-42 % Sequenzähnlichkeit mit früher beschriebenen 3-Carboxymuconat-Cycloisomerasen (vom „Typ I") aus anderen Bakterien (Parke, 1995). Das Gen *pcaB2S2* wurde aus der genomischen DNA des Stammes mit den Oligonukleotiden CMLEII-X-N und CMLEII-X-C (s. Material und Methoden, Manuskript 1) amplifiziert. Für

eine aminoterminale Fusion mit einem „His-Tag“ wurde das Gen in den Expressionsvektor pAC28 (Kholod und Mustelin 2001) zwischen *Nde*I- und *Bam*HI-Schnittstellen kloniert. Das neue konstruierte rekombinante Plasmid (pSHCMC2S2) wurde zur Transformation von *E. coli* BL21(DE3)/pLysS Star verwendet.

Mittels „partiell inverser PCR“ wurde schließlich in *H. intermedia* ca. 2 kb „downstream“ von *pcaH2G2* ein für eine 3-Carboxymuconat-Cycloisomerase kodierendes Gen identifiziert und sequenziert (*pcaB2S1*). Die folgenden Vergleiche der abgeleiteten Aminosäuresequenzen zeigten deutlich, dass die beiden putativen PcaB2-Sequenzen mit den bekannten 3-Carboxymuconat-Cycloisomerasen phylogenetisch verwandt sind. Weitergehende Sequenzvergleiche bewiesen dann, dass die beiden Typ II-Enzyme näher miteinander verwandt sind als mit den Typ I Enzymen (Abb.4).

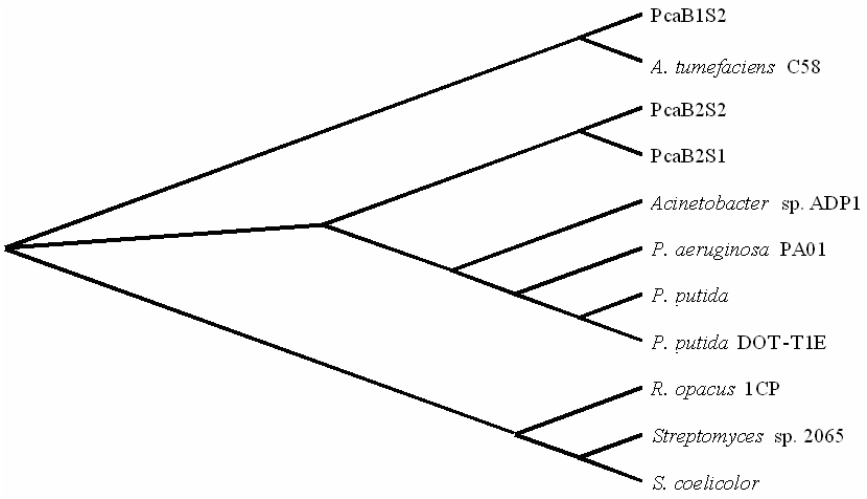

Abb. 4: Dendrogramm aus dem Vergleich der Aminosäuresequenzen verschiedener 3-Carboxymuconat-Cycloisomerasen aus verschiedenen Mikroorganismen (TreeView Win 32 Software).

Das Gen *pcaB2S1* wurde aus der genomischen DNA des Stammes *H. intermedia* mit den Oligonukleotiden pcaB2S1_X_N und pcaB2S1_X_B (s. Material und Methoden in Manuskript 1) amplifiziert. Das Amplifikat wurde in den Expressionsvektor pJOE3075 (Stumpp *et al.,* 2000) kloniert und das neue Plasmid pSBCMC2S1 genannt (Bürger, persönliche Mitteilung). Daraufhin wurde *pcaB2S1* aus dem Plasmid pSBCMC2S1 mit den Endonucleasen *Nde*I und *Bam*HI ausgeschnitten und zwischen die *Nde*I- und *Bam*HI-Schnittstellen in den Expressionsvektor pCA28 (Kholod und Mustelin 2001) ligiert. Mit dem entstehenden Plasmid (pSHCMC2S1) wurde im Folgenden *E. coli* BL21(DE3)/pLysS Star transformiert.

In den beiden konstruierten Expressionsplasmiden wurden die 3-Carboxymuconat-Cycloisomerase Typ II aus S1 bzw. S2 unter der Kontrolle des T7 Promoters mit sechs Histidin-Resten („His-Tag“) am N-terminalen Ende fusioniert. Die Proteinexpression wurde nach der Zugabe von 1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) zu den Kulturen (OD_{546nm}= 0,5 bei 30°C) in LB/Kanamycin (50µg/ml) für fünf Stunden induziert und anschließend die Induktion der Enzyme in Zellextrakten mittels SDS-PAGE analysiert. Die Größe der Untereinheiten der rekombinanten HiCMLE2 und ArCMLE2 wurde mit ca. 42 kDa. abgeschätzt (Abb. 5). Die spezifischen Aktivitäten der beiden rekombinanten CMLE2s in den Rohextrakten wurden mit 3-Carboxymuconat (3CM) photometrisch bestimmt (Ornston & Stanier, 1966). Die spezifischen Aktivitäten der ArCMLE2 bzw. HiCMLE2 lagen bei 0,5 und 0,67 U/mg.

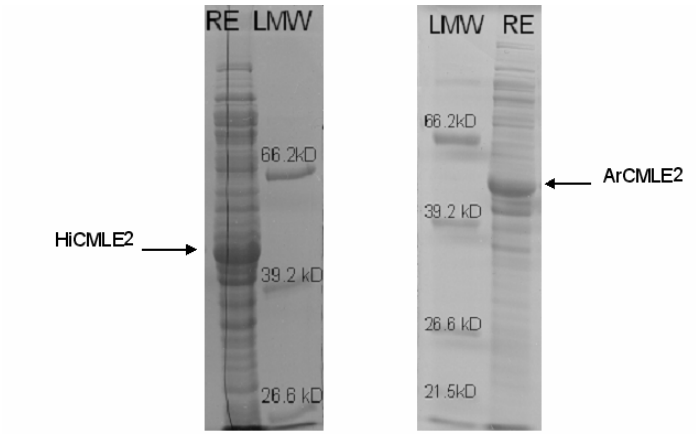

Abb. 5: SDS-PAGE-Analyse von der Induktion der HiCMLE2 und ArCMLE2. Die Zellen wurden für fünf Stunden mit IPTG (1 mM) bei 30°C inkubiert. „RE“ ist ein geklärter Rohextrakt (ca. 20 µg Protein) aus dem jeweiligen Stamm. „LMW“ ist die Standardproteinmischung (Roche Diagnostics GmbH).

2.4. Nachweis des Umsatzes von 3-Sulfomuconat (3SM) zu 4-Sulfolacton (4SL) durch die rekombinanten „Typ II“ Cycloisomerasen aus *H. intermedia* S1 und *A. radiobacter* S2

Im Folgenden wurde der Umsatz von 3-Sulfomuconat (3SM) durch ArCMLE2 und HiCMLE2 untersucht. Hierzu wurden Zellextrakte (20 µl, ca. 40 mg/ml) aus den Stämmen *E. coli* BL21(DE3)/pLysS (pSHCMC2S1) und *E. coli* BL21(DE3)/pLysS (pSHCMC2S2) mit 3-Sulfomuconat (0,5-4 mM, in Tris/HCl 50 mM, pH 8) bei 30°C (1400 rpm) inkubiert und die Reaktionen mittels HPLC analysiert. Die Messung erfolgte mit einer Fertigsäule (250 x 4 mm) mit Nucleosil C18-Material (Korngröße 5 µm) als stationärer Phase. Die mobile Phase bestand aus 98,9 % (v/v) H_2O, 1 % (v/v) Methanol, 0,1 % (v/v) H_3PO_4. Hierbei wurde

gezeigt, dass 3-Sulfomuconat (R_t= 3,5 min; λ_{max}= 209 nm) zu einem neuen Metabolit umgesetzt wurde (R_t= 4,2 min; λ_{max}= 215 nm).

Für die Identifizierung des durch den Umsatz von 3-Sulfomuconat entstehenden Metaboliten wurde der Umsatz von 3-Sulfomuconat mittels HPLC-MS/MS-Techniken analysiert. Zur chromatographischen Trennung wurde hierbei zunächst eine Ionenpaar-Chromatographie mit Tributylamin (1 mM) durchgeführt. Für die Analyse wurde Wasser/Methanol-Gradient als mobile Phase und als stationäre Phase eine C18(2)-„reversed phase"-Säule (Phenomenex, 3mm i.d. x 150 mm, 3µm Material) verwendet (s. Material und Methoden, Manuskript 1). Bei einer Wellenlänge von 254 nm wurde ein Signal (R_t= 9,8 min) identifiziert, welches mit der molekularen Masse von 3-Sulfomuconat (*m/z* 221) korreliert. Nach der Zugabe von Zellextrakten aus *E.coli* BL21(DE3)/pLysS Star(pSHCMC2S1) bzw. BL21(DE3)/pLysS Star(pSHCMC2S2) wurde 3-Sulfomuconat (R_t= 9,8 min) zu einem Produkt (R_t= 9 min) umgesetzt, welches die Masse des Molekülanions von 4-Sulfolacton (*m/z* 221) besaß. Das Produkt-Ionen-Spektrum des neuen Signals wurde aufgenommen, um weitere Strukturinformationen zu erlangen. Das so erhaltene Fragmentierungsmuster des Produktes ließ sich durch eine unterschiedliche Abfolge von Abspaltungen aus dem Molekülanion des 4-Sulfolactons erklären.

2.5. Klonierung und Expression der für die 4-Sulfolacton-Hydrolasen (SLH) kodierenden Gene aus *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2

Im Genom von *A. radiobacter* S2 folgte auf *pcaB2* ein weiterer „Open Reading Frame" (ORF). Das theoretische Genprodukt dieses ORFs besaß 35 % Sequenzidentität mit einer 2-Pyron-4,6-dicarboxylat-Hydrolase aus *Sphingomonas paucimobilis* (Masai *et al*., 1999). Auch in *H. intermedia* S1 konnte „downstream" von *pcaB2S1* ein ORF identifiziert werden, der signifikante Ähnlichkeiten mit 2-Pyron-4,6-dicarboxylat-Hydrolasen aufwies. 2-Pyron-4,6-Dicarboxylat (PDC) ist ein Intermediat in einem extradiolen Abbauweg von Protocatechuat über eine Protocatechuat-4,5-Dioxygenase Reaktion (Maruyama, 1979; Kersten *et al*., 1982) und besitzt eine Strukturähnlichkeit mit 4-Sulfolacton (Abb. 6).

Die *slh*-Gene, die für die 4-Sulfolacton-Hydrolasen kodieren, wurden aus der genomischen DNA des jeweiligen Stammes mit den Oligonukleotiden HydroS1_X_N, HydroS1_X_B, PDHS2_X_N und PDHS2_X_C (s. Material und Methoden, Manuskript 3) amplifiziert. Die Oligonukleotide fügten wiederum *Nde*I-Schnittstellen am Startkodon und *Bam*HI-Schnittstellen am Ende der Gene ein. Nach der Amplifikation wurden die PCR-Fragmente

mit den Endonucleasen *Nde*I und *Bam*HI behandelt. Für eine N-terminale Fusion mit einem „His-Tag“ wurden die Gene in den Expressionsvektor pAC28 (Kholod und Mustelin, 2001) zwischen *Nde*I- und *Bam*HI-Schnittstellen kloniert. Die neuen rekombinanten Plasmide pSHSLHS1 und pSHSLHS2 wurden für die Transformation von *E. coli* BL21(DE3)/pLysS verwendet.

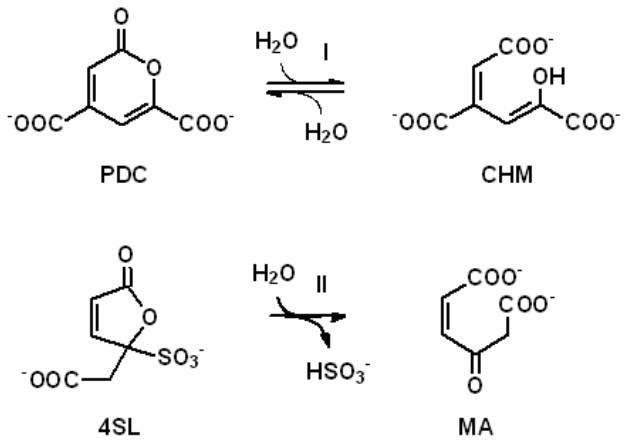

Abb. 6: Hydrolyse von 2-Pyron-4,6-dicarboxylat (PDC) zu 2-Hydroxy-4-carboxymuconat (CHM) durch eine 2-Pyron-4,6-dicarboxylat-Hydrolase (I) im Vergleich zur postulierten hydrolytischen Desulfonierung des „Sulfolactons“ (4SL) zu Maleylacetat (MA) durch die Sulfolacton-Hydrolase (II).

Die Enzyme wurden durch die Zugabe von IPTG (1mM) zu den Kulturen (300 ml, OD_{546nm}=0,5) in LB/Kanamycin (50µg/ml) Medium induziert. Die Bildung der rekombinanten 4-Sulfolacton-Hydrolasen aus den Stämmen S1 (HiSLH) und S2 (ArSLH) konnte anschließend mittels SDS-PAGE gezeigt werden. Für die Bestimmung der spezifischen Aktivitäten wurde eine photometrische Methode entwickelt, in der die Bildung von Maleylacetat bei λ = 242 nm nachgewiesen wurde. Hierdurch wurden die spezifischen Aktivitäten für die HiSLH und ArSLH in den Rohextrakten von *E. coli* BL21(DE3)/pLysS (pSHSLHS1) und *E. coli* BL21(DE3)/pLysS (pSHSLHS2) bestimmt (0,25 U/mg für HiSLH und 0,05 U/mg für ArSLH).

2.6. Aufreinigung der rekombinanten 3-Carboxymuconat-Cycloisomerasen und 4-Sulfolacton-Hydrolasen aus *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2

Die 3-Carboxymuconat-Cycloisomerasen Typ I und Typ II und die 4-Sulfolacton-Hydrolasen aus den Stämmen *H. intermedia* S1 und *A. radiobacter* S2 wurden mittels Affinitätschromatographie aufgereinigt. Hierzu wurden aus den Stämmen *E. coli* JM109 bzw. *E. coli* BL21(DE3)/pLysS, in denen die entsprechenden Gene exprimiert wurden, Zellextrakte in Tris/HCl Puffer (50 mM, pH 8.0) hergestellt. Für die Aufreinigung wurde „Ni-NTA Superflow“ Säulenmaterial (25 ml; Qiagen) in eine 25 ml FPLC-Chromatographiesäule gefüllt. Mit Hilfe einer FPLC-Anlage (Pharmacia) wurde die Säule mit 50 mM Tris/HCl (pH 8,0), 300 mM NaCl, 20 mM Imidazol und 1 mM 1,4-Dithio-D,L-threitol (DTT) äquilibriert. Ein Zellextrakt (etwa 120 mg Protein) wurde auf die Säule aufgetragen und die Säule mit etwa zwei Säulenvolumina des Äquilibrierungspuffers gewaschen. Die 3-Carboxymuconat-Cycloisomerasen und die 4-Sulfolacton-Hydrolasen wurden von der „Ni-NTA-Säule“ durch einen Elutionspuffer mit 50 mM Tris/HCl (pH 8,0), 300 mM NaCl, 1 mM DTT und unterschiedlichen Konzentrationen von Imidazol eluiert (Tab.1). Die Aufreinigung wurde jeweils mittels SDS-PAGE analysiert. Die Reinheit der aufgereinigten Enzyme lag jeweils bei etwa 90 % (Abb. 7).

Tab. 1: Aufreinigungsbedingungen der 3-Carboxymuconat-Cycloisomerasen und der 4-Sulfolacton-Hydrolasen aus *H. intermedia* S1 und *A. radiobacter* S2 bei der Affinitätschromatographie

Enzym	Imidazolkonzentration in den Elutionspuffern	Fusion mit „His-Tag“
ArCMLE1	150 mM	C-Terminal
ArCMLE2	200 mM	N-Terminal
HiCMLE2	200 mM	N-Terminal
ArSLH	100 mM	N-Terminal
HiSLH	100 mM	N-Terminal

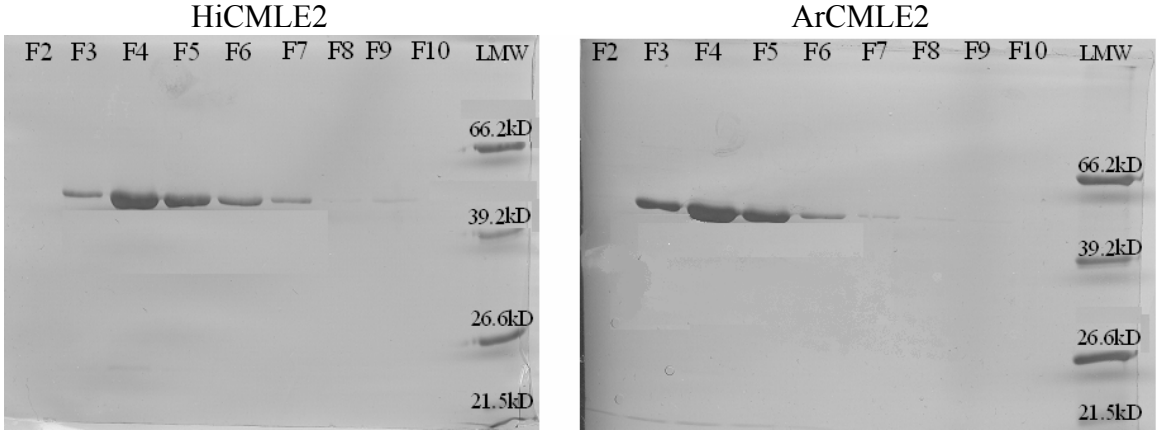

Abb.7: Aufreinigung der 3-Carboxymuconat-Cycloisomerasen Typ II aus *E. coli* BL21(DE3)(plysS)(pSHCMLES1) bzw. *E. coli* BL21(DE3)(plysS)(pSHCMLES2). Der Zellextrakt (etwa 120 mg) des jeweiligen Stamms wurde auf die „Ni-NTA"-Säule aufgetragen und die Säule wurde anschliessend mit dem Äquilibrierungspuffer (mit 50 mM Imidazolkonzentration) gewaschen. Die HiCMLE2 bzw. ArCMLE2 wurden mit dem Elutionspuffer (mit 200 mM Imidazolkonzentration) eluiert. Die Bahnen enthielten folgende Proben: „LMW" ist die Standardproteinmischung (Bio-Rad Laboratorien); in F2 bis F10 wurden die durch die Affinitätschromatographie erhaltenen Fraktionen aufgetragen; F3 bis F7 sind die Fraktionen, in denen HiCMLE2 bzw. ArCMLE2 vorhanden waren. Die Proteine wurden durch Silberfärbung sichtbar gemacht (Dodeca Silver Stain Kit Bio-Rad).

2.7. Charakterisierung der 3-Carboxymuconat-Cycloisomerasen Typ II aus *A. radiobacter* (ArCMLE2) und *H. intermedia* S1 (HiCMLE2)

Die rekombinanten ArCMLE2 und HiCMLE2 wurden mittels Affinitätschromatographie aufgereinigt und das pH-Optimum für die beiden Enzyme wurde mit 3-Carboxy-*cis,cis*-muconat bestimmt. Die Enzyme zeigten jeweils ihr pH-Optimum bei pH 8,0 in Tris/HCl-Puffer und etwa 50 % ihrer maximalen Aktivität bei pH 9,0 (Tris/HCl) oder bei pH 5,8 in Histidin/HCl-Puffer. Die beiden Cycloisomerasen wiesen bei pH 5,8 in Na/K-Phosphat-Citrat-Puffer eine niedrigere spezifische Aktivität auf als im Histidin/HCl- Puffer (etwa 1:4).
Schließlich wurde der Umsatz von 3-Sulfomuconat und 3-Carboxy-*cis,cis*-muconat durch die HiCMLE2 und ArCMLE2 verglichen. Der Umsatz von 3CM wurde photometrisch analysiert und die K_m- bzw. V_{max}-Werte für die beiden Enzyme bestimmt. Dabei stellte sich heraus, dass die V_{max}- und die K_m-Werte für die beiden Enzyme mit 3CM niedriger lagen als die für die ArCMLE1 mit 3CM bestimmten Werte (Tab. 2).
Der Umsatz von 3-Sulfomuconat wurde wiederum mittels HPLC analysiert (s. 2.4.). Verschiedene Konzentrationen (0,5 - 4 mM) von 3SM wurden mit den aufgereinigten Enzymen inkubiert. Hierbei wurden fünf Proben innerhalb von zehn Minuten genommen und

mittels HPLC analysiert (s. Material und Methoden, Manuskript 1). Die beiden CMLE2s zeigten höhere V_{max}-Werte und niedrige K_m-Werte mit dem sulfonierten Substrat im Vergleich zu dem carboxylierten Muconat (Tab. 2).

2.8. Charakterisierung der 3-Carboxymuconat-Cycloisomerase Typ I aus *A. radiobacter* S2 (ArCMLE1)

Für die Untersuchungen wurde eine mittels Affinitätschromatographie aufgereinigte Präparation der ArCMLE1 verwendet. Die Stabilität des rekombinanten Enzyms (0,05 mg/ml) wurde unter unterschiedlichen Lager-Bedingungen für jeweils 36 Tage untersucht. Hierzu wurde die ArCMLE1 bei unterschiedlichen Temperaturen in 50 mM Tris/HCl (pH 8) plus 100 mM NaCl und 0,5 mM DTT gelagert. Das Enzym war bei einer Temperatur von 4°C über den gesamten Versuchszeitraum ≥ 95 % stabil. Dagegen gingen in dem gleichen Puffersystem innerhalb von 36 Tagen etwa 50 bzw. 70 % der Aktivität bei Raumtemperatur bzw. -20°C verloren.

Anschließend wurden die grundlegenden kinetischen Daten für den Umsatz von 3CM und 3SM bestimmt. Die katalytischen Konstanten (K_m- und V_{max}-Werte) für 3CM lagen bei 0,32 ± 0,04 mM und 2270 ± 140 U/mg. Das aufgereinigte Enzym wurde mit 3-Sulfomuconat inkubiert und die Reaktion mittels HPLC analysiert. Hierbei zeigte sich überraschenderweise, dass die ArCMLE1 3-Sulfomuconat zu 4-Sulfolacton umsetzte. Die K_m- und V_{max}-Werte wurden für ArCMLE1 mit 3SM bei 11,3 ± 3,3 mM und 130 ± 30 U/mg bestimmt.

Ornston (1966) wies nach, dass die Aktivität der 3-Carboxymuconat-Cycloisomerase aus *Pseudomonas putida* PRS2000 (PpCMLE) durch 100mM Citrat gehemmt wurde. Daher wurden im Rahmen der vorliegenden Arbeit Hemmversuche mit der ArCMLE1 und Citrat und Isocitrat durchgeführt. Hierbei wurde gezeigt, dass sowohl Citrat als auch Isocitrat als nicht-kompetetive Inhibitoren fungierten: Sie erniedrigen den V_{max}-Wert, aber nicht den K_M-Wert. Die K_I-Werte wurden für Citrat bzw. Isocitrat mit 8,0 ± 2,2 mM und 7,4 ± 0,5 mM bestimmt (s. Manuskript 2). Offensichtlich binden die beiden Substanzen an das Enzym, aber außerhalb des aktiven Zentrums.

2.9. Umsatz von 3-Sulfomuconat (3SM) durch die 3-Carboxymuconat-Cycloisomerasen aus *Agrobacterium tumefaciens* A348 und *Pseudomonas putida* PRS2000

Die weiter oben beschriebenen Untersuchungen zeigten, dass ArCMLE1 in der Lage war, sowohl 3CM als auch 3SM umzusetzen. Daher wurde im Folgenden der Umsatz von 3SM durch andere 3-Carboxymuconat-Cycloisomerasen getestet, die zum Protocatechuat-

Abbauweg gehören und in früheren Arbeiten untersucht worden waren. Hierzu wurde zunächst der Stamm *Pseudomonas putida* PRS2000 auf Mineralmedium mit Protocatechuat (PC) als einziger Kohlenstoffquelle angezogen, um hierdurch die Expression der 3-Carboxymuconat-Cycloisomerase (PpCMLE1) zu induzieren (Ornston 1966). Der Zellextrakt aus *P. putida* setzte 3-Sulfomuconat (1mM) mit einer spezifischen Aktivität von 0,13 U/mg um. Im Vergleich hierzu lag die spezifische Aktivität für den gleichen Rohextrakt mit 3-Carboxy-*cis,cis*-muconat (0,1 mM) als Substrat bei 2 U/mg. Zur Bestätigung des Umsatzes von 3SM durch PpCMLE1 wurde das kodierende Gen (*pcaB*) aus der DNA des Stammes *P. putida* PRS2000 amplifiziert und in den Expressionsvektor pAC28 (Kholod und Mustelin, 2001) kloniert (s. Material und Methoden, Manuskript 1). Die PpCMLE wurde mit einer „His-Tag"-Fusion exprimiert und mittels Affinitätschromatographie aufgereinigt. Die Aktivität der aufgereinigten PpCMLE1 mit 3-Carboxy-*cis,cis*-muconat (3CM) als Substrat wurde bestimmt und lag bei 240 U/mg. Anschließend wurde 3SM (0,5, 1, 2, und 3 mM) mit der PpCMLE1 inkubiert und die Reaktion mittels HPLC analysiert. Die höchste spezifische Aktivität wurde mit 0,5 mM 3SM (8,5 U/mg) festgestellt, wohingegen die Aktivität des Enzyms bei hohen Substratkonzentrationen abnahm.

Abschließend wurde ein Zellextrakt aus dem Stamm *E. coli* JM109(pARO569) hergestellt, welcher die CMLE1 aus dem Stamm *Agrobacterium tumefaciens* A348 (AtCMLE1) exprimiert (Parke *et al.*, 2000). Trotz der hohen Sequenzähnlichkeiten zwischen ArCMLE1 und AtCMLE1 (87 %) war AtCMLE1 nicht in der Lage 3SM umzusetzen. Die spezifische Aktivität für AtCMLE1 in dem Rohextrakt betrug 8,7 U/mg mit 3CM (1mM) als Substrat (s. Manuskript 2).

Die Fähigkeit der beiden Typ I Enzyme (PpCMLE1 und ArCMLE1) sowohl 3CM als auch 3SM als Substrat zu akzeptieren deutet darauf hin, dass die CMLEs Typ I (aus dem Abbauweg von Protocatechuat) und CMLEs Typ II (aus dem Abbauweg von 4-Sulfocatechol) sich nicht strikt von einander unterscheiden.

Tab. 2: Kinetische Daten für den Umsatz von 3-Carboxy-*cis,cis*-muconat und 3-Sulfomuconat durch die 3-Carboxymuconat-Cycloisomerasen aus *H. intermedia* S1 und *A. radiobacter* S2

Enzym	MW pro Untereinheit (kDa)	Substrat	K_m (mM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	k_{cat}/K_m 3SM:3CM
HiCMLE2	48,4	3CM	0,15 ± 0,04	1,5 ± 0,31	73	486	0,73
		3SM	7,1 ± 1,9	53 ± 10	2544	358	
ArCMLE2	42,5	3CM	0,15 ± 0,07	1,3 ± 0,4	55	368	0,21
		3SM	15,3 ± 5,7	29,5 ± 9,2	1239	80	
ArCMLE1	37,4	3CM	0,32 ± 0,04	2270 ± 140	84898	265306	0,001
		3SM	11,3 ± 3,3	130 ± 30	4862	430	

2.10. Umsatz von 4-Sulfolacton zu Maleylacetat durch die 4-Sulfolacton-Hydrolasen aus *H. intermedia* S1(HiSLH) und *A. radiobacter* S2 (ArSLH)

Die 4-Sulfolacton-Hydrolasen (SLH) aus den Stämmen S1 und S2 wurden mittels Affinitätschromatographie aufgereinigt. Das Substrat 4-Sulfolacton (4SL) wurde durch den Umsatz von 3-Sulfomuconat (3SM) (4 mM, 1 ml in Tris/HCl-Puffer, pH 8) mit Hilfe der 3-Carboxymuconat-Cycloisomerase Typ I aus *A. radiobacter* (50µl, 0,5 mg/ml) präpariert. Die Reaktion wurde mittels HPLC analysiert, bis 3SM vollständig (nach 25-30 min) zu 4SL umgesetzt wurde. Die 4SL-Lösungen wurden durch Ultrafiltration („VIVASPIN 2ml Concentrator“ Membrane 10,000 MWCO PES) bei 6000 U/min für 30 min von den Proteinen abgetrennt und für die weiteren Analysen verwendet.

Die aufgereinigten Enzyme wurden mit 0,1 mM 4SL in 1 ml Tris/HCl-Puffer (50 mM, pH 8) inkubiert und die Reaktion für 10 min photometrisch in einem Wellenlängenbereich von 200- 400 nm analysiert. Hierbei wurde die Bildung eines Produkts nachgewiesen, welches ein Absorptionsmaximum bei 242 nm besaß (Abb. 8). Ein Versuch, in dem das entstehende Produkt angesäuert (pH 2) bzw. alkalisiert (pH 12) wurde, zeigte, dass das Reaktionsprodukt im sauren Bereich sein Absorptionsmaximum verlor. Dies deutete daraufhin, dass Maleylacetat das Reaktionsprodukt war (Chapman & Ribbons, 1976).

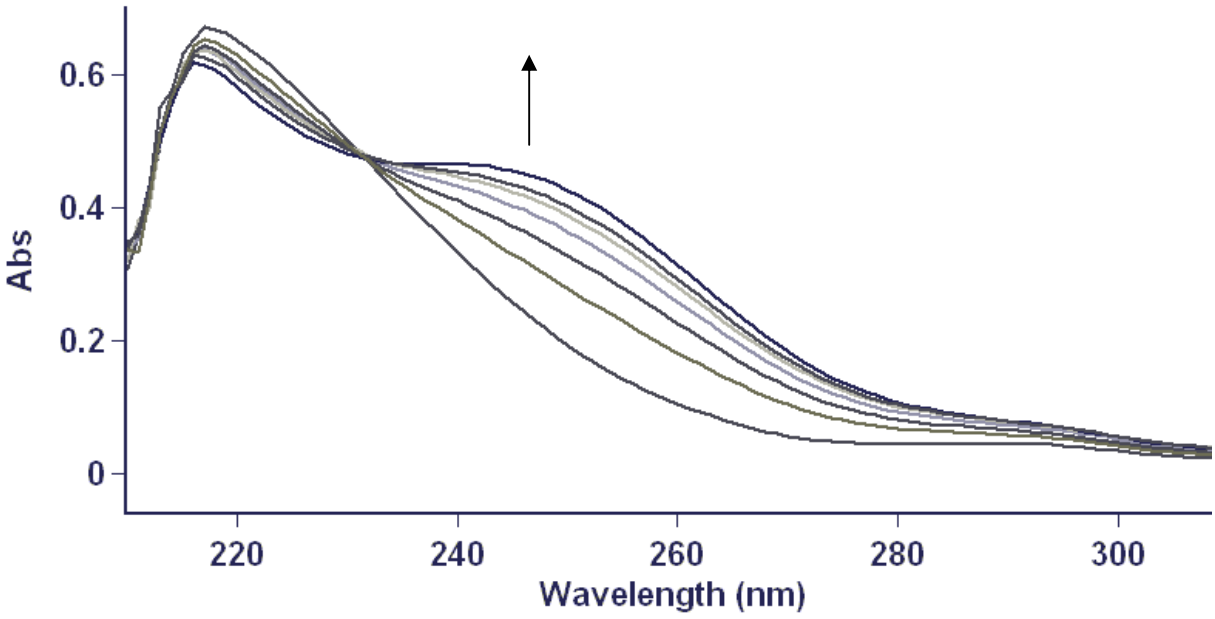

Abb. 8: Umsatz von 4-Sulfolacton durch die 4-Sulfolacton-Hydrolase aus *H. intermedia* S1 (HiSLH). 4-Sulfolacton (0,1 mM, in Tris/HCl 50 mM, pH 8) wurde mit HiSLH (5 µl, 0,5 mg/ml) bei Raumtemperatur für 6 min inkubiert. Die Reaktion wurde einmal pro Minute analysiert.

Die Entstehung von Maleylacetat wurde im Folgenden mittels HPLC bestätigt [mobile Phase: 16 % (v/v) Acetonitril, 0,1 % (v/v) TFA und 83.9 % Wasser bei einer Flussrate von 1 ml/min]. 4SL (1 mM) wurde mit der aufgereinigten 4-Sulfolacton-Hydrolase aus *H. intermedia* S1 (HiSLH) (0,05 mg/ml) in 1ml Tris/HCl-Puffer (50mM, pH 8) bei Raumtemperatur inkubiert. Die Analyse zeigte, dass 4SL (R_t = 1,3 min) nach 20 min vollständig zu einem neuen Produkt (R_t = 2,7 min) umgesetzt wurde. Ein Maleylacetat-Standard wurde durch die Alkalisierung von 4-Carboxymethylenbut-2-en-4-olid (2 mM, 2 ml) mit NaOH (1 M, 10µl) präpariert und mittels HPLC unter denselben Versuchsbedingungen analysiert. Das Produkt aus dem 4SL-Umsatz wurde durch den Vergleich mit dem authentischen Standard sowohl über die Retentionszeit als auch das UV-Spektrum als Maleylacetat identifiziert (s. Manuskript 3).
Die photometrische Analyse und die Analyse mittels HPLC bewiesen den Umsatz von 4-Sulfolacton zu Maleylacetat durch die 4-Sulfolacton-Hydrolasen aus den Stämmen S1 und S2.

2.11. Enzymatische Freisetzung von Sulfit aus 4-Sulfolacton

Die Freisetzung von Sulfit durch die HiSLH wurde nach der Methode von Johnston *et al*. (1975) mit dem Ellman's-Reagenz (5,5'-Dithio-bis-2-nitrobenzoesäure) bestimmt. Das Reagenz wurde in 0,1 M Kaliumphosphatpuffer (pH 7,0) angesetzt (1mg/ml Puffer). Eine Eichkurve wurde mit wässriger Na_2SO_3-Lösung (0-0,12 mM) bei 415 nm aufgenommen. 0, 0,02, 0,04, 0,06 und 0,08 mM 4SL (1ml, in Tris/HCl 50 mM pH 8) wurde mit HiSLH (10 µl, 0,5 mg/ml) für 10 min inkubiert (30°C, 1400 rpm). Die Proben wurden anschließend direkt mit dem Reagenz versetzt (1 ml Probe mit 0,1 ml Reagenz) und im Vergleich zu der Eichkurve wurde die Konzentration von Sulfit in jeder Probe photometrisch bestimmt. Dadurch zeigte sich, dass bei dem Umsatz der unterschiedlichen 4SL-Konzentrationen annähernd äquimolare Mengen von Sulfit freigesetzt wurden (Abb. 9).

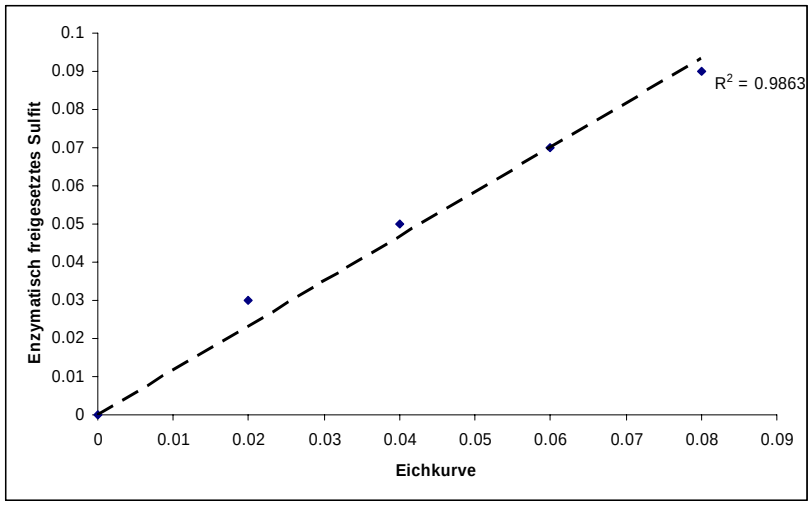

Abb. 9:. Enzymatische Freisetzung von Sulfit aus 4-Sulfolacton. Der Versuch wurde wie im Text beschrieben durchgeführt.

2.12. Charakterisierung der 4-Sulfolacton-Hydrolase aus *H. intermedia* S1 (HiSLH) und *A. radiobacter* S2 (ArSLH)

Die Enzyme wurden mit Hilfe der Affinitätschromatographie aufgereinigt. Die Molekulargewichte der HiSLH bzw. ArSLH wurden mittels Gel-Filtration mit 34,7 bzw. 32,9 kDa bestimmt. Daraus lässt sich schließen, dass die beiden 4-Sulfolacton-Hydrolasen Monomere sind, wie es auch für die 2-Pyron-4,6-dicarboxylat-Hydrolase aus *S. paucimobilis* SYK-6 festgestellt worden war (Masai *et al.*, 1999).

Sequenzvergleiche mittels „Conserved Domain Search“ (NCBI, www.ncbi.nlm.nih.gov) zeigten relative Sequenzähnlichkeiten zwischen carboxyterminalen Bereichen von 4-Sulfolacton-Hydrolasen und einer anderen Gruppe von Enzymen, die als zyklische Amidasen bezeichnet werden. Zu dieser Gruppe gehören Enzyme wie z.B. D-Hydantoinasen und Dihydropyrimidasen, die Ionen wie Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} oder Co^{2+} in ihren aktiven Zentren beinhalten (Kim & Kim, 1998). Deshalb wurde der Metallgehalt der HiSLH mittels Massenspektrometrie mit induktiv gekoppeltem Plasma (ICP-MS) (Spurenanalytisches Laboratorium, Dr. Baumann Maxhütte-Haidhof, Deutschland) bestimmt. Es wurde festgestellt, dass die Präparation einen relativ hohen Gehalt an Zn^{2+}-Ionen aufwies, welcher mit etwa 0,6 mol Zn^{2+} für 1 mol HiSLH kalkuliert wurde (s. Material und Methoden, Manuskript 3).

Abschießend wurden die grundlegenden kinetischen Daten für den Umsatz von 4-Sulfolacton durch die HiSLH und die ArSLH bestimmt. Hierzu wurden verschiedene Konzentrationen

von 4SL mit den gereinigten Enzymen bei Raumtemperatur inkubiert. Die Reaktion wurde photometrisch (λ = 242 nm) analysiert. Obwohl die HiSLH höhere V_{max}- und K_m-Werte als die ArSLH zeigte, waren die katalytischen Konstanten der beiden Enzyme sehr ähnlich (Tab. 3).

Tab. 3: Kinetische Daten für den Umsatz von 4-Sulfolacton durch die 4-Sulfolacton-Hydrolasen aus *H. intermedia* S1 and *A. radiobacter* S2.

Enzyme	MW (kDa)	K_m(mM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
HiSLH	34,7	1,9 ± 0,5	79,0 ± 18,6	2693	1417
ArSLH	32,9	0,34 ± 0,14	12,6 ± 3,3	419	1233

2.13. Versuch zum Umsatz von 4-Sulfolacton durch die 2-Pyron-4,6-dicarboxylat-Hydrolase (PDCH) aus *S. paucimobilis* SYK-6

2-Pyron-4,6-dicarboxylat (PDC) ist ein Intermediat in einem extradiolen Abbauweg von Protocatechuat über eine Protocatechuat-4,5-Dioxygenase Reaktion (Maruyama, 1979; Kersten *et al*., 1982). Aufgrund der strukturellen Ähnlichkeit zwischen 4-Sulfolacton und 2-Pyron-4,6-dicarboxylat (Abb. 6) und der ausgeprägten Sequenzähnlichkeit zwischen 4-Sulfolacton-Hydrolasen und der 2-Pyron-4,6-dicarboxylat-Hydrolase aus *S. paucimobilis* SYK-6 (PDCH), stellte sich die Frage, ob auch die PDCH in der Lage ist, 4SL umzusetzen bzw. ob die 4-Sulfolacton-Hydrolasen PDC umsetzen können. Daher wurde die Aktivität der HiSLH mit PDC getestet. Ein Rohextrakt aus *E. coli* BL21(DE3)/pLysS(pSHSLHS1) mit 4-Sulfolacton-Hydrolase-Aktivität (0,25 U/mg) wurde mit 0,1 mM PDC für 10 min inkubiert und die Reaktion photometrisch in einem Wellenlängebereich von 200-400 nm analysiert. Die HiSLH wies keine Aktivität mit PDC auf. Weiterhin wurde ein Zellextrakt aus *E.coli* JM109 (pDS15) hergestellt. Dieser Stamm exprimierte die 2-Pyron-4,6-dicarboxylat-Hydrolase aus *S. paucimobilis* SYK-6 (PDCH) (Masai *et al.* 1999). Die spezifische Aktivität der PDCH in dem Zellextrakt wurde mit 0,1 mM PDC photometrisch (λ = 315 nm) bestimmt und lag bei ca. 0,3 U/mg. Unter denselben Versuchsbedingungen wurde keine Aktivität der PDCH mit 4SL nachgewiesen. Ferner wurden mit der HiSLH Versuche zur Hemmung des Umsatzes von 4SL durch PDC durchgeführt. In 1 ml Tris/HCl- Puffer (50 mM, pH 8) wurden 4SL (0,1 mM) und PDC (0,1 mM) mit der HiSLH inkubiert und die Reaktion photometrisch

(λ = 242 nm) analysiert. Im Vergleich mit der Inkubation von 4SL mit der HiSLH unter denselben Versuchsbedingungen wurde festgestellt, dass die Anwesenheit von PDC die Aktivität der HiSLH nicht hemmte. Dies deutet darauf hin, dass PDC nicht an die HiSLH bindet.

Die Unfähigkeit der HiSLH bzw. der PDCH das jeweilige Substrat des anderen Enzyms umzusetzen, zeigte deutlich, dass die 4-Sulfolacton-Hydrolasen eine eigenständige Gruppe von Enzymen darstellt.

2.14. Bestätigung des postulierten Abbauweges von 4-Sulfocatechol durch *Hydrogenophaga intermedia* S1 und *Agrobacterium radiobacter* S2 mit Hilfe der direkten NMR-Analyse

In den früher durchgeführten Untersuchungen (Feigel, 1990) wurden die einzelnen Metabolite des 4-Sulfocatechol-Abbauweges nach Isolierung und Aufreinigung charakterisiert. Da praktisch alle beteiligten Intermediate bei der Isolation extrem instabil waren, sollte abschließend versucht werden, die postulierten Intermediate unter „naturnahen" Bedingungen zu analysieren.

Die Bestätigung des Abbauwegs von 4-Sulfocatechol (4SC) wurde mittels *in situ* ^{1}H-Kernresonanzspektroskopie (^{1}H-NMR) durchgeführt (s. Manuskript 3). Die Analyse von 4SC mit Hilfe der ^{1}H-NMR zeigte die drei erwarteten Signale der aromatischen Protonen bei 6,99 ppm (d, J=8,2 Hz), 7,27 ppm (d, J=8,2 und >2 Hz) und 7,31 ppm (d, J<2Hz) (Feigel & Knackmuss, 1988). 4SC (4 mM) wurde mit einer Protocatechuat-3,4-Dioxygenase TypII-Aktivität in einem Rohextrakt (100 µl, 26 mg/ml) von *E. coli* BL21 (DE3)(plysS)(pETS2-X-II) inkubiert. Hierbei wurde das 4SC nach 90 min vollständig umgesetzt. Das Protonenspektrum der ^{1}H-NMR-Analyse von der Lösung des Reaktionsprodukts wies drei olefinische Protonen nach, die Signale bei δ= 6,13 ppm (H5), 6,4 ppm (H4) und 6,62 ppm (H2) zeigten. Die ausgeprägte Kopplungskonstante (J = 12,4 Hz) zwischen H4 und H5 bewies, dass die beiden Protonen nicht an einem aromatischen Kern gebunden sind, was bei der chemischen Struktur von 3-Sulfomuconat der Fall ist. Nach der Zugabe von ArCMLE1 (50 µl, 0,5 mg/ml) bzw. HiCMLE2 (50 µl, 0,1 mg/ml) zu der 3SM-Lösung (1 ml, 2 mM) wurde 3SM zu einem neuen Produkt umgesetzt. Das Protonenspektrum des neuen Produktes entsprach der Struktur von 4-Sulfolacton (Feigel & Knackmuss 1993): Zwei olefinische Protonen bei δ = 6,43 und 7,87 ppm mit einer niedrigen Kopplungskonstante (J = 5,6 Hz) und zwei andere Protonen der Methylen-Gruppe bei δ= 3,21 und 3,27 ppm lassen sich mit der Struktur von 4-Sulfolacton erklären. Abschließend wurde die 4-Sulfolacton-Hydrolase aus dem Stamm S1 (HiSLH) mit der 4-Sulfolacton-Lösung inkubiert. HiSLH setzte 4SL zu

Maleylacetat (MA) als einzigem Produkt dieser Reaktion um: Zwei olefinische Protonen (δ = 6,34 und 6,47 ppm; J =12 Hz) waren offensichtlich an einer offenen Struktur und zwei Methylen-Protonen sind bei δ= 3,56 ppm nachzuweisen. Dies entsprach der H^1-NMR-Analyse von Maleylacetat in einer früheren Untersuchung (Pieper *et al.,* 2002) (Abb. 10).

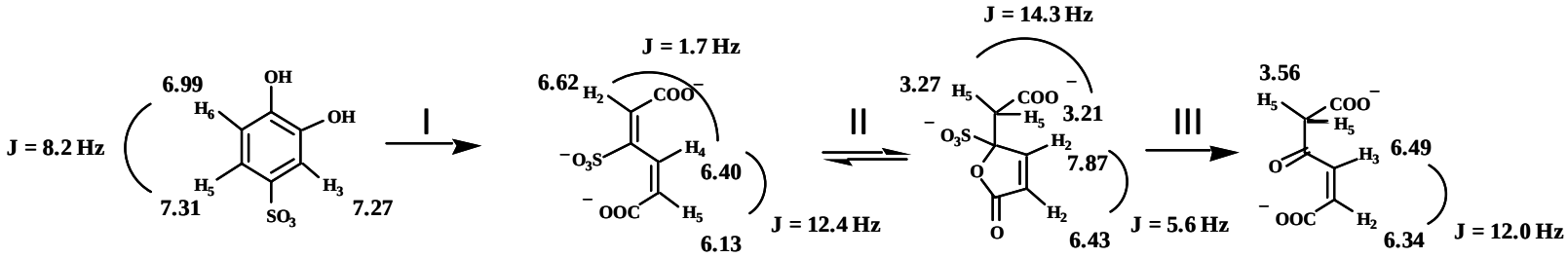

Abb. 10: ^{1}**H-NMR-Analyse des Abbauweges von 4-Sulfocatechol (4SC) durch die rekombinanten 4-Sulfocatechol abbauenden Enzyme.** I: Protocatechuat-3,4-Dioxygenase, II: 3-Carboxymuconat-Cycloisomerase und III: 4-Sulfolacton-Hydrolase aus den Stämmen S1 bzw. S2.

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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

Running title: 3-Carboxymuconate cycloisomerases from sulfanilate degrading bacteria

Subject category: Biochemistry and molecular biology

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Abbreviations: CMLE, 3-carboxy-*cis,cis*-muconate lactonizing enzyme; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; LAS, linear alkylbenzenesulfonates, DTT dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; NTA, nitrilotriacetic acid; NCBI, National Center for Biotechnology Information;

SUMMARY

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 (CMLE) and that these "type 2 enzymes" were different from the conventional CMLEs ("type I enzymes") from the protocatechuate pathway by their ability to convert 3-sulfomuconate in addition to 3-carboxy-*cis,cis*-muconate. In the present study the genes for two CMLEs were cloned from *H. intermedia S1* and *A. radiobacter* S2. In both strains, these genes were located close to the previously identified genes coding for the 4-sulfocatechol-converting enzymes. These genes were therefore tentatively identified as "type 2" enzymes (*pcaB2S1* and *pcaB2S2*) involved in the metabolism of 3-sulfomuconate. The genes were functionally expressed and the gene products shown to convert 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate. 4-Carboxymethyl-4-sulfo-but-2-en-olide (="4-sulfomuconolactone") was identified by HPLC-MS as product, which was enzymatically formed from 3-sulfomuconate. His-tagged variants of both CMLEs were purified and the basic kinetic constants determined for the purified enzymes. Furthermore, it was demonstrated that also the CMLE from the protocatechuate pathway of *Pseudomonas putida* PRS2000 converts 3-sulfomuconate.

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; Wellens, 1990; Knepper, 2002; Ruckstuhl *et al.,* 2002; Alonso *et al.,* 1999; Riediker *et al.,* 2000). The microbial degradation of aromatic sulfonic acids has mainly been studied using simple benzene- 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; Cook *et al*., 1999; Nörtemann *et al.,* 1986; Ohe *et al.* 1990; Thurnheer *et al., 1990;* Wittich *et al.,* 1988). In contrast, it was previously found that 4-aminobenzenesulfonate (sulfanilate) was initially deaminated by a coculture 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 (LAS) (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 t 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 enzymes“), which converted only protocatechuate (Feigel & Knackmuss, 1993; Hammer *et al.,* 1996). The genes coding for the protocatechuate 3,4-dioxygenases type 2 have been cloned from *H. intermedia* S1 and *A. radiobacter* S2 and significant homologies were found among the genes encoding the type 1 and type 2 enzymes (Contzen & Stolz, 2000; Contzen *et al.,* 2001).

It was 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 the cycloisomerase 3-carboxy-*cis,cis*-

muconate lactonizing enzyme (CMLE). The CMLE from *Pseudomonas putida* (PpCMLE) has previously been purified and the stereochemistry of the conversion of 3-carboxy-*cis,cis*-muconate analysed in some detail. In addition, the encoding gene has been cloned and sequenced and recently 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 involved in the metabolism of 3-sulfomuconate were characterized to verify the hypothesis that 3-sulfomuconate is indeed converted by (modified) CMLEs. This should give us further information about the specific adaptations that enable bacteria to mineralize substituted sulfonated benzenes.

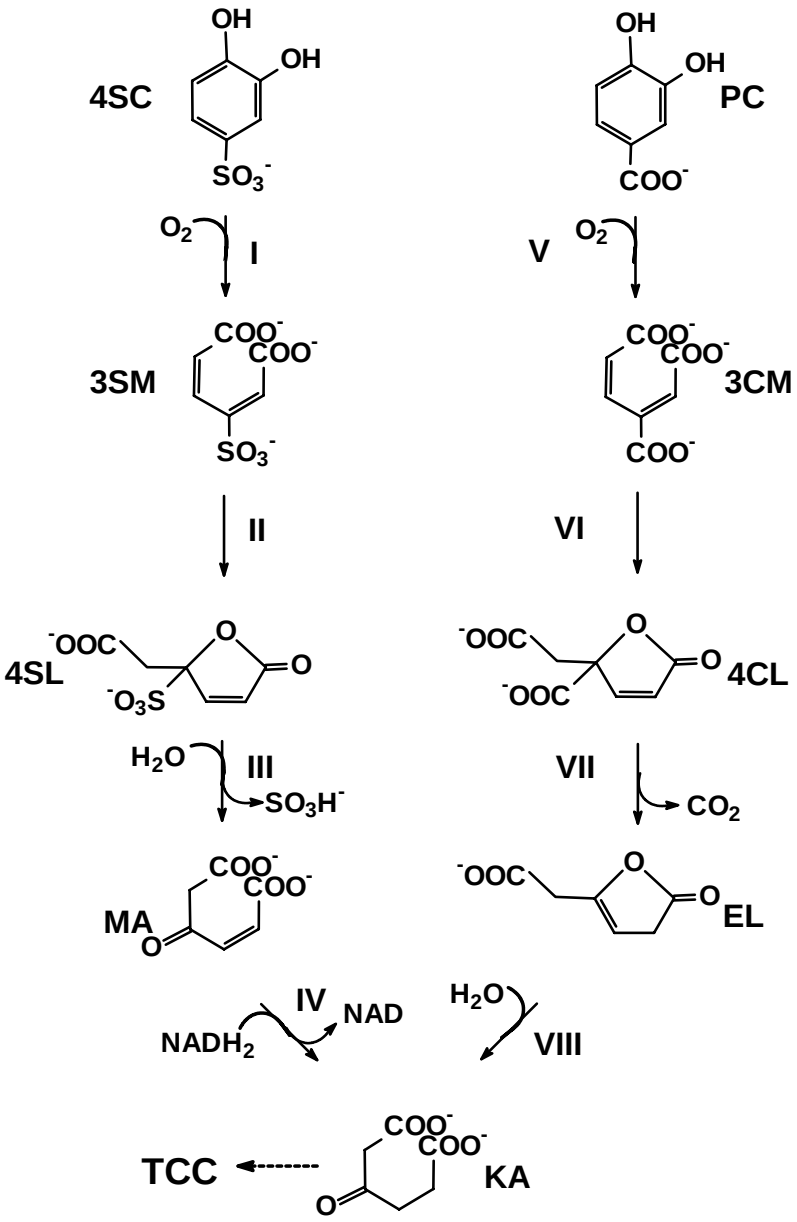

Fig 1. Proposed pathway for the degradation of 4-sulfocatechol and protocatechuate by *Agrobacterium radiobacter* S2 (Feigel and Knackmuss, 1993). Key to enzymes: I, protocatechuate 3,4-dioxygenase type II; II, 3-carboxy-*cis,cis*-muconate lactonizing enzyme type II; III, "sulfolactone" hydrolase; IV,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

METHODS

Bacterial strains and media. The isolation and characterization of *Hydrogenophaga intermedia* S1 (DSMZ 5680) and *Agrobacterium radiobacter* S2 (DSMZ 5681) has been reported before. The strains were routinely cultivated separately in SHPG-medium as previously described (Feigel & Knackmuss, 1988, 1993; Contzen *et al*., 2000).

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 rpm) in a mineral medium according to Dorn *et al.* (1974) with protocatechuate (7.5 mM) as sole source of carbon and energy.

Escherichia coli DH5α, *E. coli* JM 109, *E.coli* BL21(DE3)/pLysS and *E.coli* BL21(DE3)/pLysS Star (Invitrogen, Carlsbad, CA) were used as host strains for recombinant DNA work. *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium supplied with ampicillin (100 μg/ml) or kanamycin (50 μg/ml), 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 (Stumpp *et al., 2000*; Kholod & Mustelin, 2001). 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, Hilden, Germany). Plasmid DNA from *E. coli* DH5α was isolated with a GFX Micro Plasmid Prep kit (Pharmacia, Freiburg, Germany). Digestion of DNA with restriction endonucleases (MBI Fermentas, St. Leon-Rot, Germany), 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).

Table 1. Bacterial plasmids

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

PCR. Oligonucleotides were custom synthesized (Eurogentec, Seraing, Belgium) according to known or deduced sequences from various 3-carboxy-*cis,cis*-muconate lactonizing enzymes (CMLEs) (Table 2). PCR mixtures (50 µl) for the amplification of genomic DNA contained 50 pmol of each primer, 0.1 to 0.2 µg of genomic DNA, 0.2 mM of each deoxynucleotide triphosphate, *Taq*-DNA-polymerase (2 - 2.5 U) and the corresponding reaction buffer (Eppendorf, Hamburg, Germany).

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 *Pau*I 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*

previously obtained. The following PCR program was used: an initial denaturation (95 °C, 1 min) was followed by 30 cycles consisting of an annealing temperature of 60 °C (1 min), a polymerization step (72 °C, 4 min), and denaturation (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 of type 2 from *A. radiobacter* S2 (ArCMLE2) and *H. intermedia S1* (HiCMLE2) in *E. coli*. The gene *pcaB2S2* from *A. radiobacter* S2 was inserted into the expression vector pAC28 to generate an aminoterminally His-tagged enzyme variant (Kholod & Mustelin, 2001). The gene was amplified by PCR using the oligonucleotide primers CMLEII-X-N and CMLEII-X-C (Table 2). The primers used simultaneously introduced an *Nde*I site upstream and a *Bam*HI site downstream of the gene. The following PCR program was used: an initial denaturation (94°C, 1 min) was followed by 30 cycles consisting of an annealing temperature of 65°C (1 min), a polymerization step (72°C, 2 min), and denaturation (94°C, 1 min). The amplified product was digested with *Nde*I and *Bam*HI and cloned into *Nde*I/*Bam*HI-cut pAC28. The resulting recombinant plasmid pSHCMC2S2 was used to transform *E. coli* BL21(DE3)/pLysS Star.

HiCMLE2 was amplified using *Pfu* DNA polymerase (Stratagene, Amsterdam, Netherlands) 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 primers simultaneously introduced an *Nde*I site upstream and a *Bam*HI site downstream of *pcaB2S1*. The following PCR program was used: an initial denaturation (96 °C, 2 min) was followed by 30 cycles consisting of an annealing temperature of 63.5 °C (1 min), a polymerization step (72 °C, 2 min), and denaturation (96 °C, 30 s). The amplified product was cleaved with *Nde*I and *Bam*HI and ligated into pJOE3075 (previously cut with the same restriction enzymes). Finally, *E. coli* JM 109 was transformed with the resulting plasmid pSBCMC2S1.

Finally, a variant of the enzyme with an aminoterminal His-tag was constructed by cutting out *pcaB2S1* from pSBCMC2S1 using *Nde*I and *Bam*HI and cloning it into the expression vector pAC28, which was previously also cut with *Nde*I and *Bam*HI, giving plasmid pSHCMC2S1.

Table 2. Oligonucleotide primers used in the present study

Position	Primer name	Amino acid sequence (if relevant)	Deduced primer sequence[b] (5′→3′)
Downstream of *pcaG2* in *A. radiobacter* S2	S1-pcaG/se		caagctacgttgatcgttactgg
Consensus in CMLEs [a]	c-S1-se/c	EAALARA(Q/E)A	gaygcngcnctngcncgngcnsaygc
Consensus in CMLEs[a]	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		aacggatcctttaggcccgatgg
C-terminus HiCMLE2 + His-tag	pcaB2S1-X_HisT		ttttggatccggcccgatggttaagggc
N-terminus ArCMLE2	CMLEII-X-N		ataacatatggcattttccccccttgattc
C-terminus ArCMLE2	CMLEII-X-C		aaaaggatccttgatcagcgggtaacatttttcc
N-terminus of CMLE from *P. putida* PRS2000	pPcaB-X-N2		aaaacatatgatgaccaaccaactgttcg
C-terminus of CMLE from *P. putida* PRS2000	pPcaB-X-C2		aaaaggatcctcacccagcaccgcacgcagg

[a] 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.

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

Expression of HiCMLE2 and ArCMLE2 in *E. coli.* The CMLEs were heterologously produced as aminoterminally His-tagged enzyme variants using *E. coli* BL21(DE3)/pLysS Star(pSHCMC2S2) and *E. coli* BL21(DE3)/pLysS Star(pSHCMC2S1). The strains were grown in 300 ml of LB-medium (plus 50 μg/ml kanamycin) and the expression of the CMLEs 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 prepared. The CMLEs were purified using a Ni-NTA-column (25 ml; Quiagen, Hilden, Germany) attached to an FPLC-apparatus (Pharmacia, Uppsala, Sweden). The column was equilibrated with Tris-HCl (50 mM, pH 8.0), NaCl (300 mM), imidazole (20 mM) plus dithiothreitol (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) pooled and the elution buffer removed using a ultrafiltration unit (20 ml Concentrator, 100,000 MWCO PES; Vivascience, Hannover). Finally, the concentrated enzyme solution was diluted with Tris-HCl (50 mM, pH 8.0). This procedure resulted in enzyme preparations which appeared on silver stained gels to be greater than 99% pure for both enzymes.

Expression cloning of the CMLE from *Pseudomonas putida* PRS2000. The strain was cultivated in liquid culture with NB medium, the cells harvested by centrifugation and the DNA isolated by using an "E.Z.N.A Bacterial DNA kit" (Peqlab Biotechnologie GmbH, Erlangen, Germany). The *pcaB* gene was amplified using the primers pPcaB-X-N2 and pPcaB-X-C2 (Tab.2), which were deduced from the known nucleotide sequence of the gene (NCBI number L17082). The following PCR program was used: an initial denaturation (94 °C, 2 min) was followed by 30 cycles consisting of an annealing temperature of 65 °C (30 s), a polymerization step (72 °C, 1 min), and denaturation (94 °C, 30 s). The amplified product was cleaved with *Nde*I and *Bam*HI and cloned into pAC28 giving pSHPpCMLE. The cultivation of *E. coli* BL21(pSHPpCMLE), cell harvest and purification of the enzyme as an aminoterminally His-tagged enzyme variant were basically performed as described above for the other CMLEs.

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, Freiburg, Germany) with fluorescently labeled primers.
Sequence analysis, data base searches, and comparisons were done with the Lasergene software package, version 5 (DNASTAR Inc., Madison) and the BLAST Search at NCBI (Altschul *et al.,* 1997). The alignments of the CMLEs were obtained with the program CLUSTALX using default parameters.

Preparation of cell-free extracts. Cell suspensions in 50 mM Tris-HCl buffer, pH 8.0, were disrupted by using a French press (SLM Aminco; SLM Instruments Inc., Urbana, IL, U.S.A.) at 1.1 x 10^8 Pa. Cells and cell debris were removed by centrifugation at 100,000 g for 30 min at 4°C.

Protein estimation and enzyme assays. Protein content of cell-free extracts was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard. One unit of enzyme activity is defined as the amount of enzyme that converts 1 µmol of substrate per minute.
The 3-carboxy-*cis,cis*-muconate lactonizing activity was basically measured using the spectrophotometric assay described by Ornston & Stanier (1966). The cuvettes contained 100 µM 3-carboxy-*cis,cis*-muconate (synthesized enzymatically 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 ε_{260nm}= 7.3 $mM^{-1}cm^{-1}$.
The conversion 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 enzymatically from 4-sulfocatechol, see below). The reactions were usually monitored for 14 min and every 2 min aliquots (60 µl each) were removed and the reactions terminated in liquid nitrogen. The samples were separately thawed immediately before the HPLC analysis and the reaction rates calculated from the time-dependent decrease in the 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 a "HMW gel filtration calibration" kit (Amersham Biosciences). The subunit sizes were determined by SDS-polyacrylamide gel electrophoresis with a

"Premixed Protein Molecular Weight Marker 14.4-97.4 kDa" kit (Roche) as reference proteins.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 (Biorad).

High pressure liquid chromatography (HPLC). The turn-over of 4-sulfocatechol and 3-sulfomuconate was analyzed by reversed-phase HPLC (pumps model 510 equipped with a photo-diode array detector model 996 and Millenium Chromatography Manager 2.0, Waters Associates, Milford, Mass.). A reversed-phase column [250 × 4.0 mm (internal diameter), packed with 3 μm particles of Nucleosil C18] was used. The average flow rate was 1 ml/min. 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 average 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-mass spectrometry (LC-MS). Product identification was performed by liquid chromatography-mass spectrometry (HP1100, Agilent) coupled to a triple quadrupole mass spectrometer (Quattro LC, Micromass, Manchester, UK) using electrospray ionization in the negative ion mode. Substrate solutions before, 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 x 3 mm i.d. at 40 °C. Eluent A was H_2O/MeOH (80/20 v/v) and eluent B H_2O/MeOH (5/95 v/v) with 5 mM tributylamin and 5 mM acetic acid, each. 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, 22% B at 15 min, ready for injection after 22 min. A diode array detector and the MS 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 enzymatically using a protocatechuate dioxygenase activity (of type II) according to a method previously 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 expressed the protocatechuate 3,4-dioxygenase type II (P34OII) from *A. radiobacter* S2 under the control of a phage T7 promotor (Contzen & Stolz, 2000). The strain was grown in 300 ml of LB-medium (plus 100 µg/ml ampicillin) and the expression of the P34OII induced with IPTG (0.4 mM) as described in the „pET System Manual" (Stratagene). 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 *ca.* 40 mg ml^{-1}) were mixed with 10 ml Tris-HCl (50 mM, pH 8.0) and protocatechuate or 4-sulfocatechol were added (4 mM each). The reaction mixtures were incubated at 30°C on a laboratory shaker (100 rpm).

The cell-free extract incubated with 4-sulfocatechol were analysed simultaneously by overlay spectra and HPLC analysis. The overlay spectra demonstrated the characteristic decrease in absorbance at 280 nm due to the conversion of 4SC (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 λ_{max}= 232 nm, 281 nm) was converted to a new metabolite (R_t= 3.5 min; λ_{max}= 205 nm). The conversion of 4SC to 3-sulfomuconate was additionally verified by HPLC-MS/MS analysis. A new signal occured (R_t = 9.9 min) with a molecular anion mass $[M-H]^-$ of *m/z* 221 that corresponds to sulfomuconate ($C_6H_6O_7S$). Fragment ions detected in product ion spectra indicated the presence of one sulfonate moiety and two carboxylate groups: *m/z* 177 (M-H-CO_2), 149 (177-CO), 139 (M-H-H_2SO_3), 113 (177-CO_2), 95 (139-CO_2), 81 (HSO_3^-).

The solutions of the substituted muconates were separated from the proteins by ultrafiltration (30,000 MWCO; Vivaspin, Hannover, Germany). The resulting filtrate containing 3-carboxy- or 3-sulfomuconate was used for the enzymatic tests.

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

Nucleotide sequence accession number. The nucleotide sequences of *pcaB2S2* and *pcaB2S1* will appear in the GenBank nucleotide sequence data base under the accession numbers AY769867 and AY769868, respectively.

RESULTS

Identification, cloning and sequencing of the gene encoding the 3-carboxy-*cis,cis*-muconate lactonizing enzyme ("type 2 enzyme") from *A. radiobacter* S2 (ArCMLE2)

The genes coding for the protocatechuate 3,4-dioxygenase type 2 (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 similarities 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 gene with sequence similarities to *pcaB*s was identified (Contzen & Stolz, 2000). In the course of the present study, the DNA insert on pMCS2-2 was completely sequenced and it was found that the putative CMLE gene (*pcaB2S2*) coded for a protein of 407 amino acids (ArCMLE2) with 37-42 % sequence identity to previously described CMLEs (Fig. 2).

Identification and cloning of the gene for a putative CMLE from *H. intermedia* S1. The genes encoding for the P34OII from *H. intermedia* S1 had 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 an 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 S1 and those of the CMLEs from *Streptomyces* sp. strain 2065 (NCBI No. AAD40814), *Streptomyces coelicolor* (T35016), *Pseudomonas aeruginosa* PAO1 (NP_248921), *Pseudomonas 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, which were also present in the supposed type II enzyme from strain S2. These sequences (coding for the amino acids 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* were 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 the previous work. The respective 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 was located downstream of *pcaH2G2*. The missing part of *pcaB2S1* was finally obtained using partial inverse PCR (see materials and methods) which finally resulted in the complete sequence of *pcaB2S1*. The gene encoded a protein of 453 amino acids (HiCMLE2) with significant homology to known CMLEs over its complete length (Fig. 2).

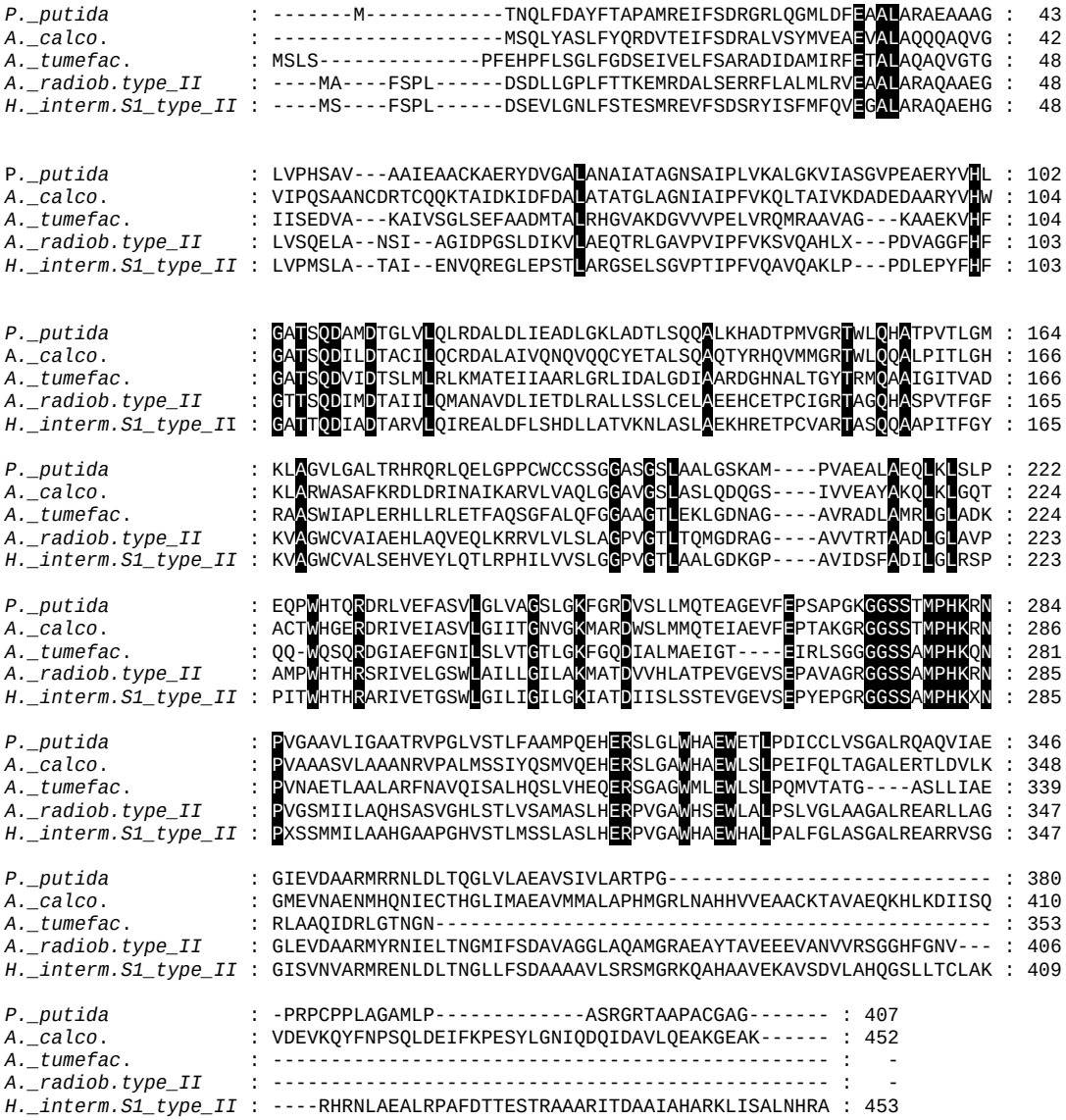

Fig. 2. **Sequence alignment of different 3-carboxy-*cis,cis*-muconate lactonizing enzymes**. Residues that are identical in all sequences are highlighted by black boxes. The accession numbers and references for the published sequences of the enzymes from *Pseudomonas putida, Acinetobacter sp.* ADP1, and *Agrobacterium tumefaciens* C58 are AF3114 (Wood *et al.*, 2001), P32427 (Williams *et al.,* 1992), and Q59092 (Kowalchuk *et al.,* 1994), and AAK88905.

Comparison of the amino acid sequences of the CMLEs from *H. intermedia S1* and *A. radiobacter S2* with each other and the 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 data base 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 (also including all sequences of presumed CMLEs from various genome sequencing projects).

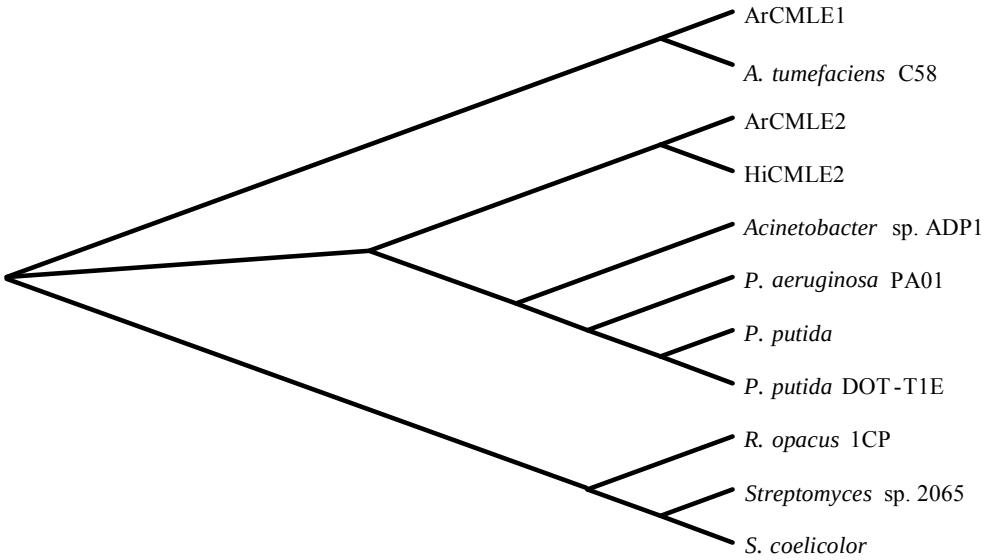

Fig. 3. **Dendrogram showing the relatedness of CMLEs from different bacterial sources.** The dendrogram was produced using the programs ClustalX and TREEVIEW (Page, 1996). The references for the sequences from *Pseudomonas putida, Acinetobacter calcoaceticus,* and *Agrobacterium tumefaciens* are given in Figure 2. The accession numbers and references for the sequences from *Bradyrhizobium japonicum, Rhodococcus opacus* 1CP, *Pseudomonas putida* DOT-T1E, *Pseudomonas 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.

Comparison of the organization of the gene clusters encodingn HiCMLE2 and ArCMLE2 to the protocatechuate gene clusters from different bacteria. The genes coding for the P34OIIs and CMLE2s were organized in *H. intermedia* S1 and *A. radiobacter* S2 differently from all known variations in the structures of protocatechuate operons. Furthermore, in both analysed strains the genes encoding the P34OIIs and the CMLE2s 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.

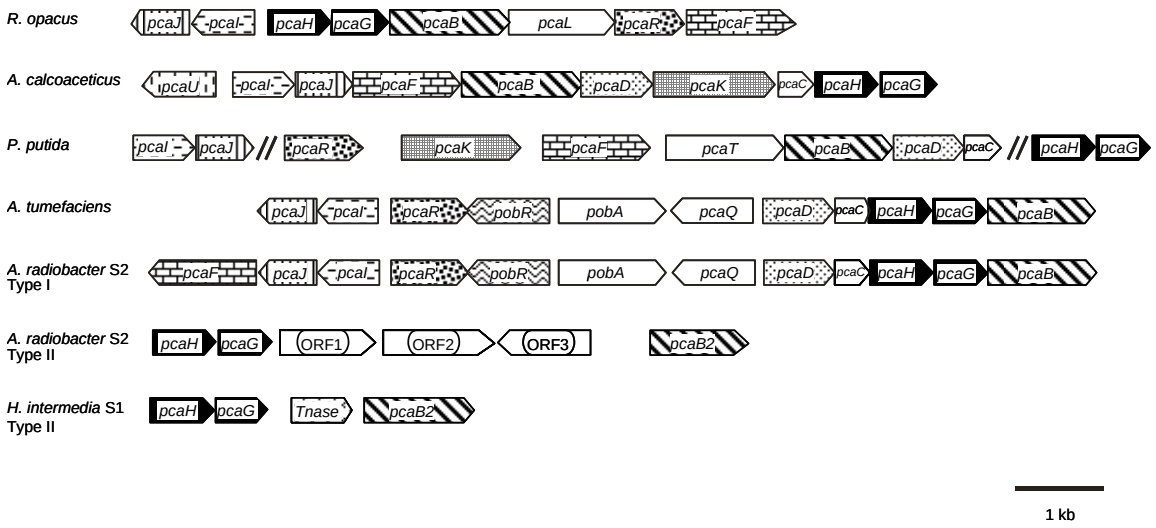

Fig. 4.Structures of the gene clusters for the catabolism of protocatechuate and 4-sulfocatechol from *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 in comparison to the protocatechuate gene clusters from *Rhodococcus opacus, Acinetobacter calcoaceticus, Pseudomonas putida* and *Agrobacterium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

Expression of the CMLE2s. The genes encoding HiCMLE2 and ArCMLE2 were cloned into the expression plasmid pAC28 under the control of the phage T7-promotor system (Kholod & Mustelin, 2001), giving plasmids pSHCMC2S1 and pSHCMC2S2. This resulted in the formation of carboxy- and aminoterminally 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 previously described by Ornston & Stanier (1966). Thus, in cell extracts from *E. coli* BL21(DE3)pLysS(pSHCMC2S1) and *E. coli* BL21(DE3)pLysS(pSHCMC2S2) specific activities of 0.67 and 0.5 U/mg of protein, respectively, were determined.

Conversion 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 absorbance at λ= 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; λ_{max}= 205 nm) was converted to a new metabolite (R_t= 4.2 min; λ_{max}= 215 nm). The subsequent analysis of the reaction by HPLC-MS/MS confirmed the conversion of 3-sulfomuconate to 4-carboxymethyl-4-sulfo-but-2-en-olide ("4-sulfomuconolactone"). A new signal was detected (R_t= 9.0 min) with *m/z* 221 for the molecular anion $[M-H]^-$. Less fragmentation than for the sulfomuconate occurred, as the cyclic lactone system remained intact and only the exocyclic functional groups were split off as neutral fragments: 177 (M-H-CO_2), 139 (M-H-H_2SO_3), 95 (139-CO_2), 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.

Conversion of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate by purified HiCMLE2 and ArCMLE2. 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 at pH 5.8. The enzymes showed significantly reduced enzymatic activities in Na/K-phosphate-citrate buffers compared to histidine-HCl buffers at the same pH as previously found for the CMLE from *P. putida* (Ornston, 1966).

Finally, the conversion of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate was compared using the purified enzymes. In the spectrophotometric tests with 3-carboxy-*cis,cis*-muconate v_{max}-values of 1.5 U/mg and 1.3 U/mg of protein were determined for HiCMLE2 and ArCMLE2, respectively (Table 3). These values were significantly lower than those previously described for the CMLE from *P. putida* (547 U/mg of protein) (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).

The conversion of 3-sulfomuconate could only be analysed by HPLC (see above). Therefore, different concentrations (0.5- 4 mM) of 3-sulfomuconate were incubated with the purified

enzymes, 5 aliquots each taken after different time intervals (2-10 min), and the conversion of 3-sulfomuconate 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).

Conversion of 3-sulfomuconate by the CMLE from *Pseudomonas putida* PRS2000. In the following it was tested if also the "archaetypical" CMLE from the protocatechuate pathway of *Pseudomomonas putida* PRS2000 (PpCMLE1) could convert 3-sulfomuconate. Cell extracts were prepared from cells of *P. putida* PRS2000 grown with protocatechuate (in order to induce the formation of PpCMLE1). The cell extracts converted 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate with specific activities of 2.0 and 0.13 U/mg of protein, respectively. In order to confirm the ability of PpCMLE1 to convert 3-sulfomuconate the encoding gene was amplified from the chromosomal DNA of *P. putida* PRS2000 and cloned into the expression vector pAC28 as described before for the two CMLE2s (see materials and methods section). The enzyme was expressed as His-tagged enzyme variant and partially purified on a Ni-NTA column to a specific activity of 240 U/mg with 3-carboxy-*cis,cis*-muconate as substrate (compared to a v_{max} reported for the purified enzyme of 457 U/mg of protein by Ornston, 1966). The same enzyme preparation was incubated with 3-sulfomuconate (0.5, 1, 2, and 3 mM) in the HPLC assay used before for the CMLE2s. 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 of protein) and a pronounced decrease in the reaction rates was observed using higher substrate concentrations.

Table 3. Kinetic data for the conversion 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

Enzyme	Source	MW per subunit (kDa)	Substrate	k_M (mM)	v_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/k_M (mM^{-1} min^{-1})	k_{cat}/k_M $_{3SM:3CM}$
HiCMLE2	*H.intermedia* 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	*A. radiobacter* 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[1]	*P. putida*	42.4	3CM	0.075	547	23200	309200	

MW molecular weight; 3 CM 3-carboxy-*cis,cis*-muconate; 3SM 3-sulfomuconate

[-1] data taken from Ornston

The data were calculated by non-linear regression using the Prism 4 software (GraphPad Software, San Diego, CA)

DISCUSSION

The results of the present study and our previous investigations of the 4-sulfocatechol-oxidizing protocatechuate 3,4-dioxygenases type II demonstrated that the degradation of 4-sulfocatechol to 4-sulfomuconolactone is catalysed by two enzymes which clearly are 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. Thus, it was found previously that the P34OIIs 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 demonstrates that both CMLEs converted carboxylated and sulfonated muconates. Furthermore, it was previously shown by multiple sequence alignments that both subunits of the P34OIIs from strain S1 and strain S2 clustered together with the corresponding subunits of the P34Os from various protocatechuate degrading organisms (Contzen & Stolz, 2000; Contzen *et al*., 2001) and here we show that also the sulfomuconate converting CMLEs were homologous to the CMLEs from protocatechuate degrading organisms.

From the sequence comparisons it was evident that the two 3-sulfomuconate converting CMLEs investigated in the present study are more closely related to each other than to the corresponding enzymes from the protocatechuate pathways of various bacteria. This was especially evident for *A. radiobacter* S2, because ArCMLE2 from this strain was more closely related to the isofunctional CMLE PcaB2S1 from the taxonomically only distantly related *Hydrogenophaga* strain (these organisms belong to the α- or β-subgroup of the *Proteobacteria*) than to the previously described CMLEs from the protocatechuate pathways of other agrobacteria (Parke, 1996; Wood *et al*., 2001). These results clearly suggest a horizontal gene transfer of ArCMLE2 from a different genetic background to strain S2.

Unfortunately, there is only very limited information available about the enzymatic characteristics of bacterial CMLEs. This is mainly due to the commercial inavailability 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 *Pseudomonas putida*) no other bacterial CMLEs have been studied regarding enzymatic characteristics in more detail.

It was a surprising observation that also PpCMLE1 which belongs to the protocatechuate pathway of *Pseudomonas putida* was able to convert 3-sulfomuconate. This suggested that

for the CMLEs no strict separation into type I enzymes (which only accept carboxylated substrates) and type II enzymes (which convert carboxylated plus sulfonated substrates) can be used as previously suggested for the 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 conversion 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 much higher v_{max}-value and a slightly lower K_M than the enzymes from *H. intermedia* S1 and *A. radiobacter* S2. It therefore seems to be much better adapted to the conversion of 3-carboxy-*cis,cis*-muconate than the "type II" enzymes studied here.

The analysis of the conversion 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 turn-over of 3-sulfomuconate are much higher than the corresponding values of HiCMLE2 and ArCMLE2. Surprisingly, it appears that the specific activities of the purified PpCMLE with 3-sulfomuconate are higher than those of HiCMLE2 and ArCMLE2. This indicates that for the conversion of 3-sulfomuconate from the point of enzyme specifity and activity no specifically adapted CMLEs are necessary. In order to analyse this apparent contradiction more accurately we are currently studying the conversion of 3-sulfomuconate by other CMLEs originating from the protocatechuate pathways of different bacteria.

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Structure and function of the 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme from the protocatechuate degradative pathway of *Agrobacterium radiobacter* S2

Running title: Structure of *Agrobacterium* Type I CMLE

Subject category: Biochemistry and molecular biology

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Abbreviations:

3CM, 3-carboxy-*cis,cis*-muconate ; 3SM, 3-sulfomuconate; CMLE, 3-carboxy-*cis,cis*-muconate lactonizing enzyme; ArCMLE1, AtCMLE1, and PpCMLE1 are 3-carboxy-*cis,cis*-muconate lactonizing enzymes from *Agrobacterium radiobacter* strain S2, *Agrobacterium tumefaciens*, and *Pseudomonas putida*; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; DTT dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; NTA, nitrilotriacetic acid; NCBI, National Center for Biotechnology Information; PEG, Polyethylene glycol; MES, 2-Morpholinoethanesulfonic acide; MPD, 2-Methyl-2,4-pentanediol; EMBL, European Molecular Biology Laboratory; ESRF, European Synchrotron Radiation Facility

SUMMARY

3-Carboxy-*cis,cis*-muconate lactonizing enzymes (CMLEs) participate in the protocatechuate branch of the 3-oxoadipate pathway of various aerobic bacteria. The gene encoding a CMLE (*pcaB1S2)* was cloned from a gene cluster involved in protocatechuate degradation by *Agrobacterium radiobacter* strain S2. This gene encoded for a CMLE of 353 amino acids - significantly smaller than all previously studied CMLEs. This enzyme, ArCMLE1, was expressed in *E. coli* and shown to convert not only 3-carboxy-*cis,cis*-muconate but also 3-sulfomuconate. ArCMLE1 was purified as a His-tagged enzyme variant and the basic catalytic constants for the conversion of 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate determined. In contrast, *A. tumefaciens* CMLE1 could not, despite 87 % sequence identity to ArCMLE1, use 3-sulfomuconate as substrate. The crystal structure of ArCMLE1 was determined at 2.2 Å resolution. Consistent with the sequence, it showed that the C-terminal domain, present in all other members of the fumarase II family, is missing in ArCMLE1. Nonetheless, both the tertiary and quaternary structure, and the structure of the active site are similar to that of *Pseudomonas putida* CMLE (PpCMLE). One principal difference is that ArCMLE1 contains an Arg, as opposed to Trp, in the active site. This indicates that activation of the carboxylic nucleophile by a hydrophobic environment is not required for lactonization, unlike earlier proposals (Yang *et al.*, 2004). We identified citrate and isocitrate as non-competitive inhibitors of ArCMLE1, and found a potential binding pocket for them on the enzyme outside the active site.

INTRODUCTION

Various aromatic compounds are degraded by bacteria under aerobic conditions *via* the catechol and protocatechuate branches of the 3-oxoadipate pathway (Stanier & Ornston, 1973; Harwood & Parales, 1996). In the protocatechuate branch of the 3-oxoadipate pathway, protocatechuate is initially oxygenolytically cleaved by protocatechuate 3,4-dioxygenase to 3-carboxy-*cis,cis*-muconate, which is then cycloisomerised by 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) to 4-carboxymuconolactone (Fig. 1).

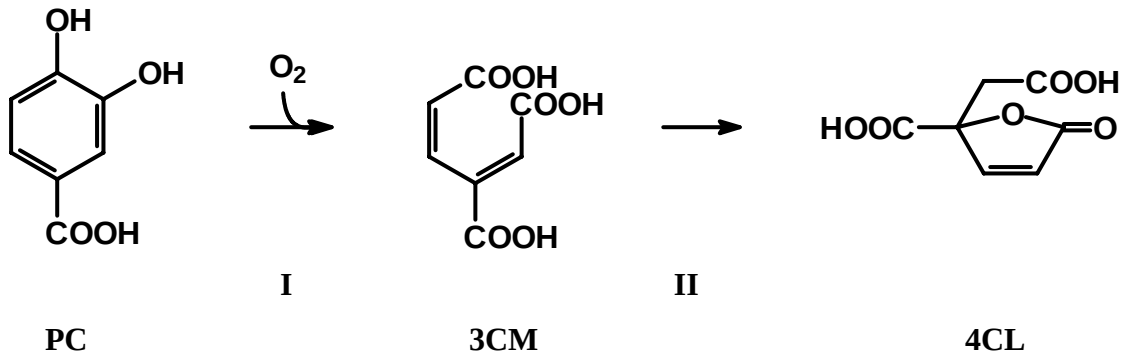

Fig. 1. **Initial steps in the protocatechuate branch of the 3-ketoadipate pathway Key to enzymes:** I, protocatechuate 3,4-dioxygenase; II, 3-carboxy-*cis,cis*-muconate lactonizing enzyme; Key to compounds: PC, protocatechuate; 3CM, 3-carboxy-*cis,cis*-muconate; 4CL, 4-carboxymuconolactone.

There is currently little information available about bacterial CMLEs from protocatechuate degradative pathways, only the CMLE from *Pseudomonas putida* (PpCMLE) has been studied in any detail. PpCMLE has been purified and characterized, the stereochemistry of the reaction analysed, and the gene encoding it cloned and sequenced (Ornston, 1966; Chari *et al*., 1987; Williams *et al*., 1992). Furthermore, its crystal structure was recently determined (Yang *et al*., 2004). Molecular and crystallographic studies demonstrated that the PpCMLE belongs to the fumarase II family of enzymes, which also includes class II fumarase, aspartase, adenylosuccinate lyase, argininosuccinate lyase and □-crystallin. All these enzymes are homotetramers with a conserved twenty-helix core. Fumarase family enzymes usually contain three different domains which intensively interact in the formation of the respective active centers (Shi *et al*., 1997; Weaver *et al*., 1998).

We are currently studying the metabolism of protocatechuate and its sulphonated structural analogue 4-sulfocatechol by a sulfanilate (4-aminobenzenesulphonate) degrading mixed

bacterial culture consisting of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 (Feigel & Knackmuss, 1988, 1993). We have cloned a gene cluster from *Agrobacterium radiobacter* S2 which appears to contain all the genes necessary for the degradation of protocatechuate to citric acid cycle intermediates (Contzen & Stolz, 2000). Similar gene clusters have also been described from *A. tumefaciens* strains A348 and C58 (Parke, 1995; Wood *et al*., 2001) and therefore appear to be characteristic for the organization of the genes involved in the degradation of protocatechuate in agrobacteria. The gene clusters contained open reading frames which were tentatively identified to encode agrobacterial CMLEs (*pcaB*) (Contzen & Stolz, 2000; Parke, 1995; Wood *et al*., 2001) downstream of the genes encoding the subunits of the protocatechuate-3,4-dioxygenase (*pcaHG*).

In the accompanying paper, we described the molecular characterization of two CMLEs from *H. intermedia* S1 and *A. radiobacter* S2, which take part in the degradation of 4-sulfocatechol by a modified version of the 3-oxoadipate pathway. These enzymes were shown to convert not only 3-carboxy-*cis,cis*-muconate but also 3-sulfomuconate, and therefore have been described as type II CMLEs. Surprisingly, it was found that also "type I" enzyme from protocatechuate pathway of *P. putida* was able to convert 3-sulfomuconate. This raised the question if also all CMLEs from the "traditional" protocatechuate degradative pathways are also able to convert 3-sulfomuconate. We therefore decided to analyse the CMLE from the protocatechuate gene cluster of *A. radiobacter* S2 because in agrobacteria the protocatechuate branch of the β-ketoadipate pathway differs significantly from other bacteria in gene organization and regulation (Parke 1995, 1996, 1997), and because of the scarcity of information available about bacterial CMLEs.

METHODS

Bacterial strains and media. *Agrobacterium radiobacter* S2 (DSMZ 5681) was cultivated in SHPG-medium as previously described (Feigel & Knackmuss, 1988, 1993). *E. coli* DH5α and *E. coli* JM 109 were used as host strains for recombinant DNA work. The *E. coli* strains were cultured in Luria-Bertani (LB) medium supplied with ampicillin (100 μg/ml). The sequence of the gene encoding the putative CMLE was determined using the previously constructed plasmid pMCS2-I-39B and *E. coli* BL21(DE3)(pLysS)(pETS2-X-II) was used for the synthesis of 3-carboxy-*cis,cis*-muconate (3CM) from protocatechuate (Contzen & Stolz, 2000).

Plasmids and DNA manipulation techniques. Plasmid pBluescript II SK (+) was used for standard cloning experiments (Alting-Mees *et al*., 1992). The plasmid vector pJOE3075 was used for high levels of gene expression (Stumpp *et al.*, 2000). Plasmid pARO569 was used for expression of the CMLE from *A. tumefaciens* CMLE (AtCMLE1). This plasmid contained a *KpnI* fragment from pARO523 (Parke, 1995) encoding AtCMLE1 under the control of the lac promotor. The plasmid was kindly provided by D. Parke (Yale University). The characteristics of all plasmids used are shown in Table 1.

Genomic DNA from *A. radiobacter* S2 was extracted using a "DNeasy Tissue Kit" (Qiagen, Hilden, Germany). Plasmid DNA from *E. coli* DH5α was isolated with a GFX Micro Plasmid Prep kit (Pharmacia, Freiburg, Germany). Digestion of DNA with restriction endonucleases (MBI Fermentas, St. Leon-Rot, Germany), electrophoresis, and ligation with T4 DNA ligase (MBI Fermentas) were performed using standard techniques (Sambrook *et al*., 1989). Transformation of *E. coli* was done as in Chung *et al*. (1989).

Oligonucleotides for PCR were custom synthesized (Eurogentec, Seraing, Belgium). PCR mixtures (50 μl) for the amplification of genomic DNA contained 100 pmol of each primer, 0.1 to 0.2 μg of genomic DNA, 0.1 mM of each deoxynucleotide triphosphate, *Taq*-DNA-polymerase (2 - 2.5 U) and the corresponding reaction buffer (Eppendorf, Hamburg, Germany).

Mutations were introduced into *pcaB1S2* by site directed mutagenesis using a QuikChange kit from Stratagene (Amsterdam, Netherlands). The mutations were verified by DNA sequencing.

Table 1: Bacterial plasmids

Plasmid	Relevant characteristics	Source or reference
pJOE3075	Expression plasmid with a rhamnose dependent promotor	Stumpp *et al*., 2000
pAC28	Expression plasmid with the T7 promotor	Kholod & Mustelin, 2001
pETS2-X-II	Expression of *pcaH2G2* from *A. radiobacter* S2 under the control of the T7 promotor	Contzen *et al*., 2001
pMCS2-I-39B	*pcaG1* and *pcaB1S2* from *A. radiobacter* S2 in pBluescript II SK(+)	Contzen & Stolz, 2000
pSHCMC1S2	*pcaB1S2* from *A. radiobacter* S2 in pJOE3075 (encodes ArCMLE1 with a carboxyterminal His-tag)	this study
pARO569	CMLE from *A. tumefaciens* under the control of the lac promotor	D. Parke (Yale University)

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, Freiburg, Germany) with fluorescently labeled primers.

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

Expression of ArCMLE1 and AtCMLE1 in *E. coli*. For recombinant expression, *pcaB1S2* from *A. radiobacter* S2 was inserted into the expression vector pJOE3075, to produce a

carboxy-terminal His-tagged enzyme, as follows (Stumpp *et al*., 2000) . The DNA segments encompassing *pcaB1S2* were amplified by PCR using the primers CMLEI-X-N (ATA-ACA-TAT-GAG-CCT-TTC-CCC-CTT-CGA-AC) and CMLEI-His-C (AAA-GGA-TCC-GCT-TTC-GTC-AGC-CCC-CAG-C) thus introducing *Nde*I sites upstream and *Bam*HI sites downstream of the gene. The following PCR program was used: an initial denaturation (94° C, 1 min) was followed by 30 cycles consisting of an annealing temperature of 65° C (1 min), a polymerization step (72° C, 2 min), and denaturation (94° C, 1 min). The amplified product containing *pcaB1S2* was then cleaved with *Nde*I and *Bam*HI and cloned into pJOE3075 (also cut with *Nde*I and *Bam*HI). The resulting recombinant plasmid pSHCMC1S2 was subsequently used to transform *E. coli* JM 109. Expression was induced by adding 0.2% (w/v) L-rhamnose to the culture (OD_{546nm}=0.2 - 0.3) in LB/ampicillin medium. Induction was performed for 6 h at 30 °C.

E. coli JM109(pARO569) was used for expression of AtCMLE1**.** The recombinant strain was grown in 150 ml of LB-medium plus chloramphenicol (10 □g/ml) to an optical density (OD_{546nm}) of 0.5, 1 mM IPTG was added, and the cells grown till they reached an OD_{546nm} of about 5. Finally, the cells were harvested by centrifugation and cell extracts prepared.

Preparation of cell-free extracts. Cell suspensions in 50 mM Tris-HCl buffer, pH 8.0, were disrupted with a French press (SLM Aminco; SLM Instruments Inc., Urbana, IL, U.S.A.) at 1.1 x 10^8 Pa. Cells and cell debris were removed by centrifugation at 100,000 *g* for 30 min at 4°C.

Purification of His-tagged ArCMLE1. Cell extracts of *E. coli* JM109(pSHCMC1S2) were prepared in Tris/HCl buffer (50 mM, pH 8.0) as described above. The "Ni-NTA Superflow" column material (25 ml; Qiagen) was transferred to an empty 25 ml-FPLC chromatography column. The filled column was attached to an FPLC apparatus (Pharmacia) and equilibrated with a buffer system (pH 8.0) consisting of 50 mM Tris/HCl, 300 mM NaCl, 20 mM imidazole, and 1 mM of 1,4,-dithio-D,L-threitol (DTT). The cell extracts (about 120 mg of protein) were applied to the column and the column was washed with 1-2 column volumes of the equilibration buffer. ArCMLE1 was then eluted using a buffer system (pH 8.0) consisting of Tris/HCl (50 mM), NaCl (300 mM), DTT (1 mM), and 150 mM imidazole. 5 ml fractions

were collected and the fraction showing enzymatic activity (usually fractions 3 and 4) used for enzymatic studies and crystallization.

Protein analysis and enzyme assays. The protein content of cell-free extracts was determined by Bradford assay (1976) with bovine serum albumin as standard. The purity of the protein preparation was assessed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (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 (Biorad).

Enzyme activities were measured with 3-carboxy-*cis,cis*-muconate and 3-sulfomuconate with the enzymatically synthesized compounds. The activity with 3-carboxy-*cis,cis*-muconate (3CM) was measured spectrophotometrically using substrate concentrations from 0.01 to 0.6 mM and the activity with 3-sulphomuconate (3SM) was measured by HPLC (see accompanying paper for further details). One unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate per minute. Kinetic studies of inhibition by isocitrate and citrate were done using similar concentrations of 3CM and 5 to 25 mM inhibitor. All kinetic data were fit using GraphPad Prism v4.0 (San Diego, CA).

Crystallization and data collection. Purified ArCMLE1 was buffer-exchanged into 10 mM Tris-HCl pH 7.5 plus 1 mM DTT using a Sephadex G-25 column (PD-10 Amersham Biosciences) and the protein concentrated to 20 mg/ml by ultrafiltration (Centricon, Millipore, Billerica, Mass., USA). The protein concentration was measured spectrophotometrically using a calculated extinction coefficient of 24040 $M^{-1}cm^{-1}$ at 280 nm. ArCMLE1 crystallized in two different crystal forms in sitting drops from either 100 mm cacodylate pH 6.5, 15 % PEG 8000, 100 mM ammonium sulphate ($P2_1$ crystal form) or 100 mM MES pH 6.5, 15 % PEG 8000, 100 mM ammonium sulphate, 3 % MPD ($P2_12_12_1$ crystal form). The crystallization drop consisted of 1 μl of protein solution (10 mg/ml), 1 μl of 4 mM 3-sulfomuconate and 1 μl of well solution. Crystals appeared after several weeks of incubation at room temperature.

For data collection, crystals were quickly dipped in well solution supplemented with 20 % glycerol and flash cooled to 100 K in a stream of boil-off nitrogen gas. The $P2_1$ and $P2_12_12_1$

crystals diffracted to 2.2 Å and 2.6 Å resolution, respectively. An initial low resolution data set (3.2 Å) of the $P2_1$ form was collected at EMBL Hamburg outstation on beamline BW7A. The diffraction quality of the crystals improved over time and the highest resolution data were collected from four months old crystals at ESRF on beamline ID14-3 (Table 2).

Structure solution and refinement. The structure was initially solved using the 3.2 Å data collected at Hamburg on the $P2_1$ form with program Phaser (Storoni *et al.,* 2004) using a tetrameric model constructed from the previously solved CMLE structure(Yang *et al.*, 2004; PDB id: 1RE5), including only residues 3-350. Phaser was able to locate three tetramers in the asymmetric unit. We used Bodil (Lehtonen *et al.*, 2004) to convert the molecular replacement solution into a rough model of ArCMLE1. Only sidechains were replaced and no further energy minimization was applied before refinement against the experimental data. The structure was refined with CNS 1.1 (Brünger *et al.*, 1998) using strict noncrystallographic symmetry constraints between the twelve monomers. This resulted in R factors of 0.266 for R_{work} and 0.284 for R_{free} (5% of the reflections). We then used a monomer of this preliminary structure as a model for Phaser (Storoni *et al.*, 2004) to solve the 2.2 Å $P2_1$ structure. The 2.6 Å $P2_12_12_1$ structure was solved with Molrep (Vagin & Teplyakov 1997) from the $P2_1$ structure using a refined tetramer as a model. Both the $P2_1$ and $P2_12_12_1$ structures contain three homotetramers in the asymmetric unit. The higher resolution structures were refined with Refmac (Murshudov, 1997) and manual model correction was done with Coot (Emsley & Cowtan, 2004). Initially medium NCS restraints were applied between monomers but during the final stages of refinement the restraints were completely released. Also, during the last refinement cycle, a conservative TLS refinement was used: each TLS group consisted of a biological tetramer. TLS refinement decreased the R-factors by 1 % (R_{work}) and 0.4 % (R_{free}) for the $P2_1$ structure and by 1.3/0.6 % for the $P2_12_12_1$ structure. The final R-factors for the models are 18.8/23.6 % for the $P2_1$ and 19.9/26.1 % for the $P2_12_12_1$ structures. The geometry of the models is acceptable (Table 2)**.**

Table 2: Summary of data processing and refinement. Values in parenthesis are for the highest resolution shell.

	$P2_1$ crystal form	$P2_12_12_1$ crystal form
Resolution (highest shell) (Å)	20-2.2 (2.3-2.2)	20-2.6 (2.7-2.6)
Wavelength (Å)	0.931	0.931
Number of observations	857321 (108008)	446581 (42109)
Number of unique reflections	219955 (27503)	133154 (13747)
Space group	$P2_1$	$P2_12_12_1$
Unit-cell parameters (Å,º)	a=90.86 b=208.51 c=123.93 β=108.35	a=94.03 b=205.32 c=235.74
Completeness (%)	99.5 (99.5)	94.6 (92.4)
R_{merge} § (%)	8.6 (45.4)	10.1 (47.9)
I/σ(I)	12.1 (3.0)	10.5 (2.6)
R factor † (%)	18.8	20.0
R free ‡ (%)	23.6	26.2
Number of atoms per asymmetric unit		
Protein	30578	30583
Water	1252	825
Other	87	25
B-factors ($Å^2$)		
Protein	33.3	49.0
Water	28.7	33.0
Other	63.6	74.2
R.m.s. deviations		
Bond lengths (Å)	0.010	0.011
Bond angles (°)	1.457	1.455
Ramachandran plot		
Most favoured regions (%)	92.7	91.3
Other allowed regions (%)	7.3	8.7

§ $R_{merge} = \Sigma_i | I_i - \langle I \rangle | / \Sigma \langle I \rangle$, where I is an individual intensity measurement and ⟨I⟩ is the average intensity for this reflection with summation over all data. † R factor is defined as $\Sigma| |F_{obs}| - |F_{calc}| | / \Sigma |F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure-factor amplitudes, respectively. ‡ R free is the R factor for the test set (5 % of the data).

The final models for all 12 independent monomers in the $P2_1$ asymmetric unit contained residues 2-268 and 281-350; some of the monomers contained either Gly269 (E, G, H, J and K chains) or Gly269-Gly270 (B and F chains) as well. Monomers ABCD, EFGH and IJKL form biological tetramers. For two chains in the $P2_12_12_1$ structure, we were able to build even more of the missing loop; chain A contains also residues Gly269-Gly270-Gly271 and Lys279-Gln280 and chain D contains Gly269-Gly270-Gly271 and Pro280-Lys279-Gln280. At 2.2 Å, we were able to build alternate conformations for residues Glu22, Ser63, Asn188 (on the surface) and Leu302 (hydrophobic patch within monomer). Each of the 12 independent monomers contained at least one residue with alternate conformations; no monomer contained all four.

Modelling of *A. tumefaciens* CMLE. A homology model of AtCMLE was based on the $P2_1$ structure of ArCMLE1. The ArCMLE1 monomer A was used as a model in Bodil (Lehtonen *et al.,* 2004) and sidechains were changed, and loops and C-terminus truncated according to the sequence alignment. Loop 269-280 was removed from the automatically generated model, as it is not defined in the crystal structures. The biological tetramer was then generated based on the ABCD tetramer of $P2_1$ structure described here.

Accession numbers. The nucleotide sequences of *pcaB1S2* will appear in the GenBank nucleotide sequence data base under the accession number AY769866. Coordinates and structure factors for the $P2_1$ and $P2_12_12_1$ structures of ArCMLE1 are deposited in the Protein Databank with accession codes 2FEL and 2FEN, respectively. Coordinates for the homology model are available upon request from the authors.

Chemicals: The chemicals used were obtained from Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), and Sigma (Neu-Ulm, Germany).

RESULTS

Cloning and sequencing of the *pcaB1S2* gene. In the gene-cluster from strain S2 an ORF was identified directly downstream of the genes coding for the protocatechuate 3,4-dioxygenase (P34OI) (*pcaH1G1*). It showed significant sequence homology to known 3-carboxy-*cis,cis*-muconate lactonizing enzymes (CMLEs). The sequence of the gene encoding the putative CMLE was determined using the previously constructed plasmid pMCS2-I-39B (Contzen & Stolz, 2000). The gene was designated as *pcaB1S2* (= *pcaB* from the type I gene cluster of strain S2). The gene encoded a protein (ArCMLE1) consisting of 353 amino acids with a GC-content of 60.6 %. ArCMLE1 showed the highest degree of sequence homology to presumed CMLEs from *A. tumefaciens* (87 % sequence identity to *A. tumefaciens* CMLE; *13*). The sequences of the CMLE1 from members of the *Rhizobiales*, such as ArCMLE1 and AtCMLE1, are significantly shorter than the isofunctional enzymes from other bacteria (Fig. 2).

Expression of ArCMLE1. Comparison of the sequence of ArCMLE1 with that of the recently published crystal structure of the CMLE from *P. putida* (Yang *et al*., 2004) suggested that the carboxyterminal enzyme domain was completely missing in the agrobacterial enzymes. Therefore, plasmid pSHCMC1S2 was constructed by amplifying *pcaB1S2* and cloning the gene into the expression vector pJOE3075 (see Materials and Methods). After the addition of rhamnose an intense new peptide band was observed in crude extracts of *E. coli* JM 109(pSHCMC1S2). The size of the additional band was estimated by SDS-PAGE as about 37 kDa.

Conversion of 3-carboxy-*cis,cis*-muconate. *E. coli* JM 109(pSHCMC1S2) was grown in LB/ampicillin medium plus rhamnose. Cell extracts were prepared and the CMLE activities in the cell extracts tested using the spectrophotometric enzyme assay originally described by Ornston & Stanier (1966). The overlay spectra demonstrated that the cell extracts from *E.coli* JM 109(pSHCMC1S2) converted *3-carboxy-cis,cis-muconate* (3CM) to 4-carboxymuconolactone and a CMLE activity of 9.4 U/mg of protein was determined. In contrast, no conversion of 3CM was found in cell extracts of *E. coli* JM109 which did not harbour the plasmid.

Fig. 2

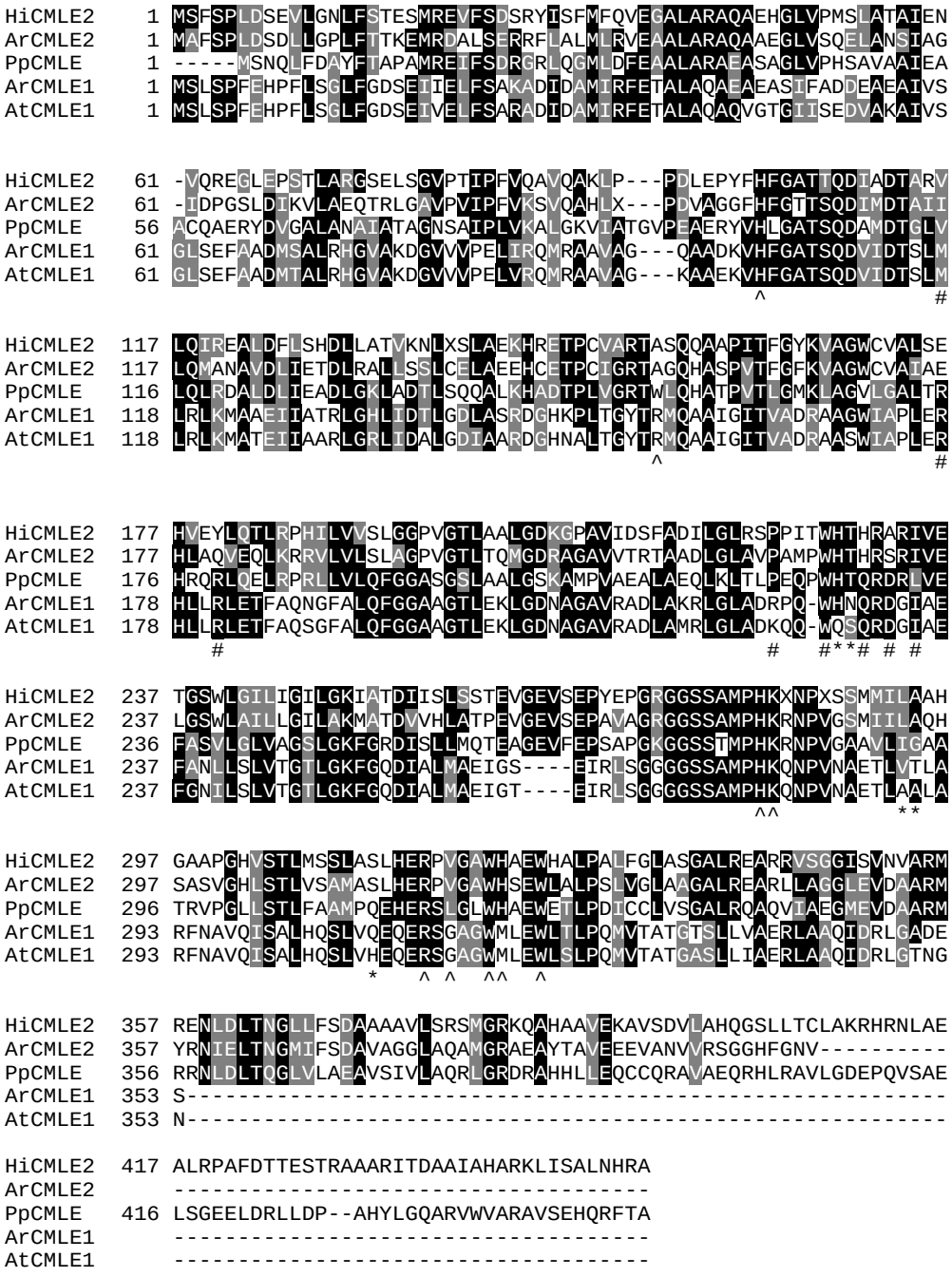

Fig. 2. **Sequence alignment of different 3-carboxy-*cis,cis*-muconate lactonizing enzymes.** Residues that are identical in all sequences are highlighted by black boxes. The residues forming the active site are marked with ^, residues forming the potential allosteric site are marked with # and the residues that have changed in AtCMLE and disabolished the capability to lactonize 3SM are marked with *. The accession numbers of the sequences are: HiCMLE2 AY769868, ArCMLE2 AY769867, PpCMLE AAN67002, ArCMLE1 AY769866 and AtCMLE1 AAF34266.

Kinetic parameters for *A. radiobacter* S2 CMLE1. ArCMLE1 was purified by affinity chromatography on Ni-NTA matrix. The kinetic constants were determined. The purified enzyme (0.05 mg/ml) was almost completely stable during 36 days of storage at 4°C in 50 mM Tris/HCl plus 100 mM NaCl and 0.5 mM DTT. In contrast, after storage for the same time at room temperature or at -20°C in the same buffer system, it lost more than 50% of its activity.

ArCMLE1 has a pH-optimum of 6.0-7.0 and K_M and V_{max}-values of 0.32 ± 0.04 mM and 2270 ± 140 U/mg for 3CM. The purified enzyme was also incubated with 3-sulfomuconate (3SM) and the conversion of the substrate analysed by HPLC. Surprisingly, the enzyme converted 3SM to 4-sulphomuconolactone as previously observed for the CMLEs from the 4-sulfocatechol degradative pathway ("type II enzymes") (see accompanying paper). As HPLC analysis is slower, only a rough estimate of the reaction constants could be obtained; the K_M was about 11.3 ± 3.3 mM m and the V_{max} at about 130 ± 30 U/mg.

Ornston (1966) showed that PpCMLE was inhibited by 100 mM citrate. A very similar effect was also observed for ArCMLE1, suggesting that citrate exhibited a specific effect on this group of enzymes. Because citrate shows some structural resemblance to 3CM, we measured kinetics in the presence of citrate, with isocitrate as a negative control, in order to find out the nature of the inhibition. Surprisingly both citrate and isocitrate acted as non-competetive inhibitors (Fig. 3), lowering only the V_{max} but not the K_M of the lactonization reaction. The K_I-value is 18.0±2.2 (±sem) mM for citrate and 7.4 ±0.5 mM for isocitrate. Isocitrate and citrate thus do not bind to the active site, but to somewhere else in the protein.

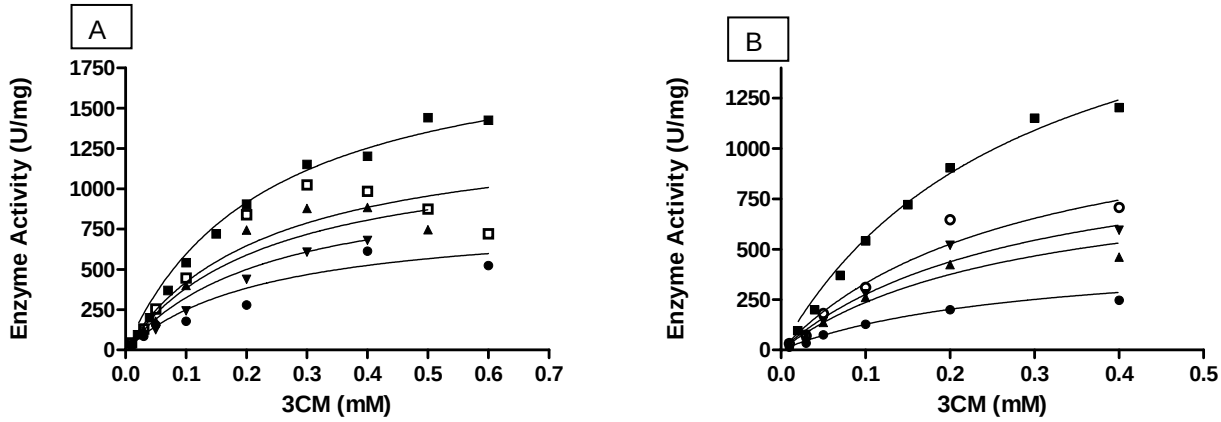

Fig. 3. Inhibition of the 3-carboxy-*cis,cis*-muconate lactonizing enzyme from *A. radiobacter* S2 (ArCMLE1) by citrate (A) and isocitrate (B). The reaction mixtures contained in a total volume of 1 ml 67 µmol Na/K-phosphate buffer (pH 6.5) and the indicated concentrations of 3-carboxy-*cis,cis*-muconate (■). In the left part of the figure 7.5 mM (□), 10 mM (▲), 15 mM (▼), or 25 mM (●) citrate were added and in the right part 5 mM (○), 7.5 mM (▼), 10 mM (▲) or 25 mM (●) isocitrate. The individual reactions were monitored for 30 s.

Conversion of 3-SM by the CMLEs from *Agrobacterium tumefaciens* A348. The results obtained previously for PpCMLE and for ArCMLE1 suggested that the type 1 enzymes from "traditional" protocatechuate pathways could also convert 3-sulfomuconate to sulphomuconolactone. Therefore, it was in addition tested if cell extracts from *E. coli* JM109(pARO569) which expressed the CMLE from *Agrobacterium tumefaciens* (AtCMLE1) convert 3SM. The cell extracts from *E. coli* JM109(pARO569) had rather high specific activity with 3CM (8.7 U/mg), but showed no activity with 3SM. This was surprising, because AtCMLE1 has 87% sequence identity to ArCMLE1**.**

Site directed mutagenesis of ArCMLE1. From the crystal structure of the *P. putida* CMLE (Yang *et al*., 2004) and sequence comparisons with other members of the fumarase II family, it was proposed that Trp153, Lys282, and Arg315 are involved in catalysis. The alignment of the small CMLEs from different members of the *Rhizobiales* with these sequences demonstrated that in *A. radiobacter* S2 (and the other member of *Rhizobiales*) the amino acid residues corresponding to Lys282 and Arg315 were conserved, but that Trp153 in *P. putida* was always replaced by an Arg. To analyse if the amino acid at this position is important for the enzymatic reaction, the R155A mutation was introduced into ArCMLE1 by site specific mutagenesis. The resulting mutant enzyme did not show any activity with 3CM or with 3SM.

A gel-filtration experiment demonstrated that the mutation did not alter the tetrameric behaviour of the protein, indicating that the mutation affected the catalytic machinery directly, rather than the oligomeric state of the enzyme.

Overall structure. The variation in size and the observed amino acid modification between PpCMLE and ArCMLE suggested important differences between the two enzymes. Therefore, the crystal structure of the monomeric as well as tetrameric structure of *A. radiobacter* S2 ArCMLE1 is very similar to PpCMLE (root mean square deviation (rmsd) of 1.6 Å for 1192 C_α atoms of a tetramer and 1.44 for 306 C_α atoms of a monomer). This indicates that not only is the monomer structure conserved, but also the quaternary structure of the tetramer. Despite this, ArCMLE1 completely lacks the C-terminal domain and the C-terminal helix that in PpCMLE returns to the core structure (Figure 4). The lack of this helix, although it seems to be needed for monomer interactions in PpCMLE, nonetheless does not affect the overall oligomeric organization. In both ArCMLE1 structures the asymmetric unit consists of twelve monomers that form three physiological tetramers. Monomers generally contain residues 2-268 and 281-350 (see Materials and Methods); the missing 8-13 residues (depending on monomer) form a loop covering the active site. In some of the monomers in the $P2_12_12_1$ structure, we have more density for the loop and were able to model a few more residues, including the Lys279 that points into the active site.

Monomers in the $P2_1$ structure are also very similar to each other; the rmsd/C□ is 0.19 - 0.54 Å with an average of 0.31 Å. In the $P2_12_12_1$ structure, the deviation range is 0.29 - 0.58 Å with an average of 0.39 Å. The deviations between the monomers in the $P2_12_12_1$ structure are larger than in the higher resolution $P2_1$ structure, especially monomer J in $P2_12_12_1$, which has an average deviation from the other monomers of 0.49 Å/C□. This is presumably due to crystal contacts; monomer J contains several regions with poorly-defined electron density, in particular loop 41-66, which lies in the interface between the EFGH and IJKL tetramers, and residues 89-104, which form a helix-loop structure on the surface. Deviations between monomers of the two structures are in the same range as within independent structures. Monomer H from the $P2_1$ structure differs clearly the most from the others with an rmsd/C□ of 0.38-0.54 Å (depending on the comparison structure). This difference is largely a result of changes at the C-terminus of helix 51-65, caused by crystal contacts with an adjacent

asymmetric unit. In monomer I this region is also different than in all other monomers, although it does not participate in crystal contacts. The B-factors (Table I) for this region are similar in all the monomers and so the differences appear to be caused by discrete independent conformations, rather than continuous flexibility.

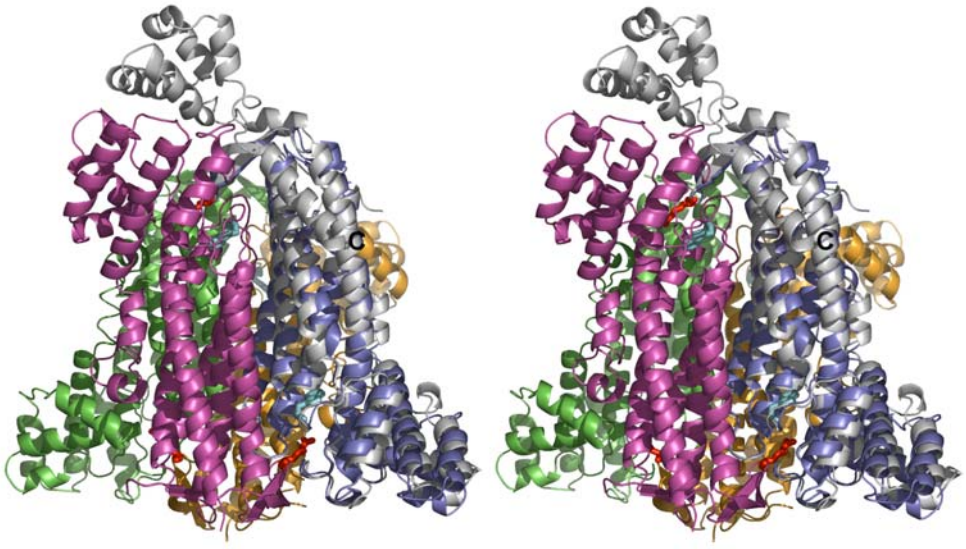

Fig. 4. Tetrameric structure of 3-carboxy-*cis,cis*-muconate lactonizing enzyme from *A. radiobacter* S2. Subunits forming the tetramer are coloured differently (A: blue, B: magenta, C: green and D:orange). Monomer of PcaB (grey, pdb code 1RE5) is superpositioned over a ArCMLE1 monomer ($P2_1$ structure) in order to show the missing C-terminal domain and the last C-terminal helix. The whole tetrameric structure was used for superpositioning. To indicate the locations of the active and allosteric sites: Arg155 is shown in red as a stick model in the active site and Trp227 in cyan in the potential allosteric site. C-terminus of PpCMLE is labelled, in order to indicate the C-terminal helix missing in ArCMLE1. Image was created with Pymol (Delano 2002).

Potential allosteric binding site. We observed unexplained continuous electron density near Trp227, which forms the base of a binding site formed from two adjacent monomers (AB, BA, CD, DC). The density was present in all monomers at about 4.5-6.5 σ in the final σA weighted (Fo-Fc) electron density map (Figure 5). The hydrophobic portion of the AB pocket is formed by $Trp227^A$, $Ile234^A$ and $Met117^A$ (the superscript here and below indicating which monomer). The hydrophilic part of the pocket probably binds negative charge because it is formed by $Arg224^A$, $Gln230^A$, $Arg177^B$ and $Arg181^B$. $Asp232^A$ forms ion-pairs with $Arg177^B$ and $Arg181^B$. The residues come from A helix 109-145, the N-terminus of A-helix 231-260

and the preceeding loop 220^{A}-230^{A}, and from the B monomer helix 165-187. We could not fill the density with water molecules nor with any of the crystallisation nor purification components (Tris, MES, Cacodylate, DTT or MPD). The shape of the density did not seem to change when we co-crystallized with 40 mM citrate; nor did it depend on whether we added 3SM to the crystallization drop or not. This is therefore most likely the result of a small molecule which binds tightly to the protein during expression or purification. Electrospray ionization mass spectrometry coupled to a liquid chromatrography (LC-ESI-MS) experiments to identify the molecule were, unfortunately, inconclusive (data not shown).

The AB pocket (*i.e* mostly monomer A including $Trp227^{A}$) is 13 Å from the DAB active site (measured from the Cα of Arg312; Figure 4), 42 Å from active site ABC, 36 Å from active site BCD and 42 Å from active site CDA. It is therefore possible that binding to this site modulates the activity in the active site. The effect could be transmitted through a loop 224-231 that lies below the active site arginine (Arg312) which appears to be essential for substrate binding (see discussion). Furthermore, sequence alignment suggests that the 224-231 loop may be important in modifying the substrate spectrum of ArCMLE1 (see below). Residues Arg224 is not conserved in other CMLEs, and Arg177 and Arg181 are not conserved in the "type II" CMLEs (Fig. 2). This suggests that these enzymes may not have the binding pocket we have identified. Furthermore, even in PpCMLE this potential allosteric binding pocket is filled mainly by the Arg232 sidechain, which in ArCMLE1 and AtCMLE1 is glycine.

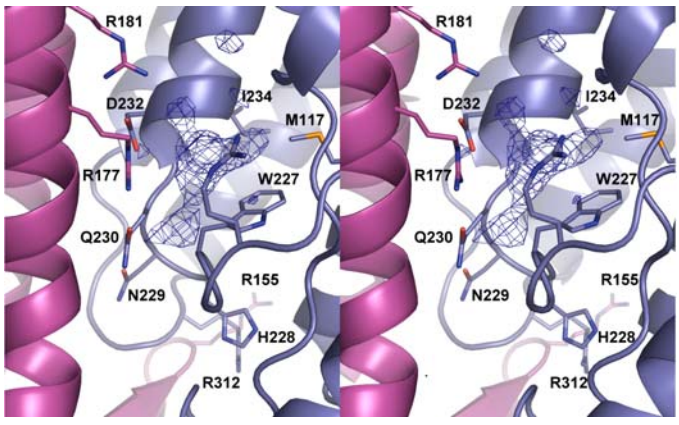

Fig. 5. 12-fold NCS averaged density in the σA weighted Fo-Fc electron density map near Trp227 of monomer A in $P2_1$ structure contoured at 7 σ. NCS averaging was done with Coot (Emsley & Cowtan 2004). Residues in monomer A are coloured in blue and residues of monomer B in magenta. Image was created with Pymol (Delano 2002).

Active site. Each of the four active sites per tetramer is formed from three monomers, as mentioned above. Below, we describe the geometry in the DAB active site, though the others are essentially identical; the chain identities merely permute. We describe this as the "A" active site, as chain A forms the base of the active site. Although 3SM was used in the crystallization mixture, we did not see it in the active site. Instead, a chloride ion could be modelled into some of the active sites where spherical electron density near $Arg155^{B}$ indicated a molecule heavier than water. The active site of ArCMLE1 shows important differences in comparison with PpCMLE (Figure 6A). $Trp153^{B}$ was proposed to be a critical residue in the catalytic mechanism of PpCMLE (Yang *et al.*, 2004), but in ArCMLE1 this residue is replaced by $Arg155^{B}$. $Arg155^{B}$ (and $Trp153^{B}$ in PpCMLE) also participates in monomer-monomer interactions and there are changes in the surrounding residues correlated with the Trp-Arg change. In PpCMLE, $Trp153^{B}$ forms a hydrophobic interaction with $Leu317^{A}$. This leucine is replaced by glycine in ArCMLE1, thus creating room for $Glu286^{D}$, which forms a salt bridge with $Arg155^{B}$. The equivalent of $Glu286^{D}$ in PpCMLE is $Ala289^{D}$. On the opposite side of the active site, PpCMLE $His321^{A}$ is replaced by $Met318^{A}$ (Fig. 6A). Overall the "top" of the active site (Fig. 6A) maintains a positive-hydrophobic axis, with one side positive and the other side hydrophobic, but the identity of the residues is completely changed. The change from PpCMLE $Leu317^{A}$ to ArCMLE1 $Gly314^{A}$ together with a reorientation of the C-monomer main chain due to a peptide-flip at 314^{A} makes room for the Arg-Glu pair mentioned above (Fig. 6A).

Yang *et al*. (2004) located a citrate molecule at very high B-factor in one of the active sites of a tetramer and, as in our $P2_12_12_1$ structure, they could see a few more residues of the loop, including the lysine pointing towards the active site. The binding mode of citrate in PpCMLE structure agrees with our structure in the sense that it binds to the active site arginine ($Arg312^{A}$), which is in a similar conformation in both structures. Our preliminary docking results (data not shown) also indicate that one of the carboxylates of citrate would be actually bound to the $Arg155^{B}$ in ArCMLE1.

Below the active site $Trp317^{A}$ and $Trp321^{A}$ (Figure 6A), there is a cavity filled with 14 ordered water molecules (Figure 6B). This cavity is in the interface between monomers A and D and is surrounded mainly with hydrophobic residues ($Pro5^{D}$, $His8^{D}$, $Phe10^{D}$, $Leu11^{D}$,

Phe24^{A}, Val82^{A}, Ile112^{A}, Leu116^{A}, Leu120^{A}, Ile234^{A}, Leu324^{A}, Trp317^{A}, Trp321^{A} and Pro325^{A}). The water cavity near the active site may be important in creating flexibility required for the enzyme catalysis.

Modeling of *A. tumefaciens* CMLE. We constructed a homology model of AtCMLE1 (87 % identical to ArCMLE1) to understand why it does not lactonise 3SM, unlike ArCMLE1. The sequence of the loop covering the active site is identical in both enzymes and therefore is unlikely to contribute to this difference in specificity. There are no changes in the active site, but there are a few changes in the region between the active site and the "allosteric binding site" identified above. As both sites are formed by multiple monomers, we refer here to the DAB active site, which is close to the AB "allosteric binding site". His228^{A} and Asn229^{A} of ArCMLE1 are Asn and Ser, respectively, in AtCMLE1. Asn229 of ArCMLE1 is not conserved in other enzymes that degrade 3SM (Figure 2), but only AtCMLE1 has Asn at position 228. His228^{A} in ArCMLE1 is very close to Arg312^{A} (Fig. 6B), which presumably binds substrate. Although the residues are not hydrogen bonded, the removal of positive charge next Arg312^{A} might have an effect on substrate binding. Furthermore, His228^{A} is in the same loop as Arg224^{A}, which is part of the "allosteric binding site" 224-232 loop and adjacent to the Trp227 forming the basis of this binding site (see above). Finally, ArCMLE1 Gln308^{A} is replaced by His308^{A} and Val289^{D} and Thr290^{D} on helix 283-308 are both mutated to Ala in AtCMLE1. These changes might affect the flexibility at the back of the active site.

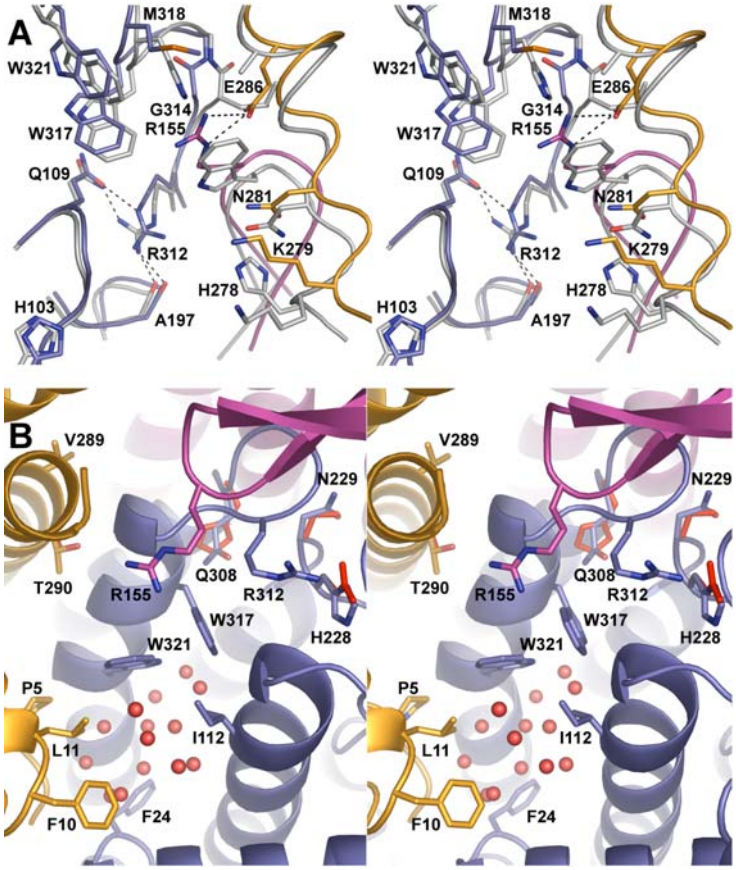

Fig. 6. A) Comparison of the active sites. ArCMLE1 active site is in grey and PpCMLE active site is in blue (chain A), magenta (chain B) and orange (chain D). Residues are labelled according to ArCMLE1 sequence. Hydrogen bonds of active site arginines (Arg155 and Arg312) are shown in dashed lines. Sidechain of His278 is not visible in ArCMLE1 structure. Figure was made from the coordinates of the $P2_12_12_1$ structure of ArCMLE1 and of PpCMLE (pdb code: 1RE5).

B) Water cavity below the active site. View is from the top of the active site. Water molecules are show as red spheres. Colouring of the chains is same as in figure 6A. Some of the residues surrounding the water cavity are shown. Residues that differ in the AtCMLE1 homology model (H228N, N229S, V289A, T290A and Q308H) are shown in red. Figure was made based on the higher resolution $P2_1$ structure. Both figures were made with Pymol (Delano 2002).

DISCUSSION

ArCMLE1 is the first truncated CMLE that has been characterized; indeed, it is the first truncated fumarase-fold enzyme. Its C-terminal truncation includes the whole of the C-terminal domain, including the very last helix which, in homologous enzymes like PpCMLE (Yang *et al.*, 2004) (and ArCMLE2; see accompanying paper) folds back into the protein core and participates in monomer-monomer interactions. Sequence analysis (Fig. 2) suggested this to be the case and our structure demonstrates that, indeed, it is so. The C-terminal domain is thus not required for formation of the oligomeric structure; the rmsd between PpCMLE and ArCMLE1 is 1.6 Å for the tetramer and 1.4 Å for the monomer. In addition, it seems clear that the C-terminal domain is not important in catalysis; the truncation increased k_{cat} to over 10^5 min^{-1} (versus values of 0.067-23x10^3 min^{-1} for other enzymes; accompanying paper, Table 3). ArCMLE is thus the fastest CMLE so far characterised. If the rate-determining step is product release, as is often the case for non-control point enzymes (Albery & Knowles, 1976) the increase in k_{cat} may reflect faster binding and debinding because the "upper jaw" of the active site is missing. There is no significant difference in the K_M for 3CM, except for PpCMLE, which binds 3CM more tightly than the other enzymes we have studied (accompanying paper; Table 3).

Type I enzymes were believed to show no or only very limited activity with 3SM (Feigel & Knackmuss, 1993), but our results demonstrate that ArCMLE1 not only catalyses the lactonization of 3SM, but does so even faster than the type II counterparts. The K_m values with 3SM for both *A. radiobacter* CMLEs and also the type II enzyme from *H. intermedia* are relatively poor (7- 15 mM). The ratio of k_{cat}/K_m for 3SM versus 3CM suggests that a distinction can be made between type I II enzymes, which degrade 3CM alone, and type II enzymes, with improved enzymatic specificity for 3SM. For instance ArCMLE1 has a relative k_{cat}/K_m for 3SM of 0.0016, while the type II enzymes *H. intermedia* CMLE2 and *A. radiobacter* CMLE2 have relative k_{cat}/K_m for 3SM of 0.73 and 0.21 respectively (see accompanying paper). Nonetheless, ArCMLE1 catalyses the lactonisination of 3SM better in terms of k_{cat} and k_{cat}/K_m than any of the type II enzymes studied except HiCMLE2 (see accompanying paper). The basis for 3SM-specificity is still unclear.

Although ArCMLE1 can lactonise 3SM, AtCMLE1 can not and so homology modeling should allow one to identify the specific amino acid changes that affect substrate specificity. Surprisingly, there are no changes in residues in the active site cavity, so all changes in

catalytic activity are due to secondary changes outside the active site. We have identified four possible amino acid changes; His228Asn, Val289^{A}Ala, Thr290^{A}Ala and Gln308^{A}His (ArCMLE1→ AtCMLE1) (Fig. 2). His228Asn may reduce the overall positive charge in the active site, while the Val289^{A}Ala, Thr290^{A}Ala and Gln308^{A}His changes may affect the conformation or flexibility of the active site. These small changes may thus prevent binding of 3SM in a catalytically competent manner. The situation is analogous to that in the muconate lactonizing enzyme from *P. putida* and *Pseudomonas* sp. P51 chloromuconate lactonising enzyme. In these enzymes, changes that are not part of the active site affect conformational flexibility in the active site and thus whether dehalogenation happens on the enzyme or not. This dehalogenation requires a rotation of the newly formed lactone ring by 180° (Kajander *et al*., 2003).

Our inhibition experiments with citrate and isocitrate showed that they are non-competitive inhibitors of ArCMLE1, despite the structural resemblance to the substrate molecule. They do not compete with substrate, but bind somewhere else in the protein and modulate its activity. Intriguingly, we located a possible binding site 13 Å away from the active site, separated from the active site only by Trp227, which forms the base of the allosteric site and by His228 and Asn229, which also appear to cause the difference in substrate specificity between ArCMLE1 and AtCMLE1. The binding site contains three arginines (Arg224, Arg177 and Arg181) but, although the density superficially resembles that of citrate, we were not able to confidently satisfy the density with citrate-like molecules nor to detect a small molecule ligand by LC-ESI-MS.

The type I enzyme from *Pseudomonas putida* (PpCMLE), which binds 3CM four times tighter than ArCMLE1, contains a tryptophan residue in the active site (Trp153). Yang and coworkers (Yang *et al.*, 2004) proposed that the reaction starts by nucleophilic attack of the oxygen of the 6-CO_2^- group on position C3 of 3CM to form an aci-intermediate, which would be stabilized by PpCMLE Arg315. The reaction then proceeds by proton transfer from the general base (PpCMLE Lys282) to the aci-intermediate to form 4-carboxymuconolactone. The hydrophobic environment created by Trp153 has been proposed to activate the nucleophilic carboxylic group of the substrate (Yang *et al*., 2004).

Trp153 is, in ArCMLE1, Arg155, and so the same activation can not occur in this enzyme. We also made the Arg155Ala variant, as Ala is found at this position in type II enzymes (Fig.

2). This variant was completely inactive, which is not surprising as $Arg155^{B}$ forms a salt bridge with $Glu286^{D}$. Two changes can be predicted in the mutant. First, there is an increase in the negative charge in the active site and, second, breaking the salt bridge would alter the quaternary structure of the protein and so the active site architecture. Both changes would lead to an inactive enzyme.

In some type I enzymes the residue corresponding to the Arg155 is Leu (Fig. 2) while it is Ala in the type II ArCMLE2 and HiCMLE2 (Fig. 2; accompanying paper). This sequence variability, together with the structural role of the Arg/Trp (see above) makes it unlikely that this residue is required for catalysis as previously suggested (Yang *et al*., 2004). A positive charge appears, however, to be required on the "right" (Fig. 6A) of the active site. When the residue corresponding to ArCMLE1 Arg155 is hydrophobic, the disordered loop covering the active site contains a positive charge at position Gly270 and Gln280 (Fig. 2). Another change in comparison with the other CMLEs is at position 275, where PpCMLE has a Thr instead of Ala; this might cause the ten fold tighter K_m for 3CM observed in PpCMLE. All these residues, Lys273, Thr278 and Arg283, are not visible in the PpCMLE model and therefore we cannot assess their roles. Finally, the fumarase class II charge relay pair (His141-Glu275) is replaced by Trp153-Val283 in PpCMLE and by Arg155-Glu286 in ArCMLE1. Although the charge properties are thus preserved in ArCMLE1 (though not in PpCMLE), Arg is a very poor general acid and so is unlikely to participate in the reaction mechanism.

Preliminary docking results suggest that the binding mode proposed by Yang *et al*. (2004) is possible for ArCMLE1 as well. The lowest energy docking results, which show direct interaction between the $6\text{-}CO_2^-$ to $Arg155^{B}$, are probably not physiological because this residue is involved in stabilising the interaction with chain D. If substrate binds as in Yang *et al*. (2004), $Arg312^{A}$ could help withdraw electrons from the $1\text{-}CO_2^{2-}$ group to make the 3-position more electrophilic; it would also stabilise the aci-carboxylate intermediate. This would allow Lys279, as proposed by Yang and coworkers (Yang *et al*., 2004), to act as the general acid. There are, however, candidates for the general acid other than Lys279: conserved histidines His103 and His278. The latter is also part of the mobile loop and in PpCMLE it points towards the active site (Fig 6A).

Our structure, mutagenesis and comparison studies indicate that the exact mechanism of ArCMLE1 and other CMLEs is far from settled. The role of the interactions around Arg155 remains unclear, as does the importance of nucleophile (6-CO_2^-) activation. In addition, it is interesting that, in MLEs as a rule, residues outside the active site cavity have significant effects on catalysis, in some cases changing the reaction stereochemistry (Kajander *et al.*, 2003) and here affect reactions specificity dramatically. The structural basis for these effects remains to be explored.

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4-Sulfomuconolactone hydrolases from *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2

Running title: 4-Sulfomuconolactone hydrolases from sulfanilate degrading bacteria

Subject category: Physiology and metabolism

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Abbreviations: CMLE, 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; LAS, linear alkylbenzenesulfonates, DTT dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; NTA, nitrilotriacetic acid; NCBI, National Center for Biotechnology Information;

ABSTRACT

The 4-carboxymethylen-4-sulfo-but-2-en-olide hydrolases ("4-sulfomuconolactone hydrolases") from *Hydrogenophaga intermedia* strain S1 and *Agrobacterium radiobacter* strain S2 are part of a modified protocatechuate pathway responsible for the degradation of 4-sulfocatechol. The encoding genes were identified in both strains "down-stream" of the genes encoding the 3-sulfomuconate converting 3-carboxy-*cis*,*cis*-muconate lactonizing enzymes which catalyse the formation of the 4-sulfomuconolactone. The genes encoding the 4-sulfomuconolactone hydrolases were cloned and sequenced. The deduced amino acid sequences of the 4-sulfomuconolactone hydrolases demonstrated the highest degree of sequence identity to 2-pyrone-4,6-dicarboxylate hydrolases which take part in the extradiol ("meta-") cleavage of protocatechuate. The 4-sulfomuconolactone hydrolases did not convert 2-pyrone-4,6-dicarboxylate and the 2-pyrone-4,6-dicarboxylate hydrolase from *Sphingomonas paucimobilis* SYK-6 did not convert 4-sulfomuconolactone. Nevertheless, the presence of four highly conserved histidine residues in the 4-sulfomuconolactone hydrolases and the 2-pyrone-4,6-dicarboxylate hydrolases and some further sequence homologies suggested that both enzymes belong together with hydantoinases, allontoinases, dihydropyriminidases, and dihydroorotases to a group of enzymes previously believed only to catalyse the hydrolysis of cylic amides ("cyclic amidases"). The 4-sulfomuconolactone hydrolases were heterologously expressed as His-tagged enzyme variants. Gel filtration experiments suggested that the enzymes are present as monomers in aqueous solution. The fundamental enzyme parameters for the conversion of 4-sulfomuconolactone were compared for both enzymes and the reactions analysed by in-situ NMR. The purified enzyme presumably contained Zn^{2+}-ions in its active center.

INTRODUCTION

Aromatic sulfonic acids are 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). Several laboratory and environmental studies have shown that the presence of a sulfonic acid group usually significantly decreases the rates of biodegradation of this class of compounds compared to unsulfonated structural analogous (Alexander & Lustigman, 1966; Alonso *et al*., 1999; Knepper, 2002; Riediker *et al*., 2000; Ruckstuhl *et al*., 2002; Wellens, 1990).

Our laboratory is currently studying the degradation of 4-aminobenzenesulfonate (sulfanilate) by a coculture of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2. In this mixed bacterial culture, sulfanilate is initially oxygenolytically deaminated by strain S1 to 4-sulfocatechol. 4-Sulfocatechol is then converted in both strains to 3-sulfomuconate by specifically adapted forms of protocatechuate 3,4-dioxygenases. In the next enzymatic step, 3-sulfomuconate is converted in a cycloisomerization reaction to a sulfonated lactone (Fig. 1). The ability of the 4-sulfocatechol and 3-sulfomuconate transforming enzymes also to convert their carboxylated structural counter-parts (protocatechuate and 3-carboxy-*cis,cis*-muconate) and sequence comparisons clearly demonstrated that both enzymes originate from the protocatechuate branch of the β-ketoadipate pathway (Feigel & Knackmuss, 1988, 1993; Hammer *et al*., 1996; Contzen & Stolz, 2000; Contzen *et al.*, 2001; Halak *et al*., manuscript submitted). The product formed from 3-sulfomuconate by the 3-carboxy-*cis,cis*-muconate lactonizing enzymes has been identified as 4-carboxymethylene-4-sulfo-but-2-en-olide ("4-sulfomuconolactone") (Feigel & Knackmuss, 1993; Halak *et al*., manuscript submitted). This metabolite structurally resembles its equivalent from the protocatechuate pathway ("4-carboxymuconolactone"= 4-carboxymethylene-4-carboxy-but-2-en-olide) (Fig. 1). Nevertheless, the further metabolism of the 4-carboxymuconolactone and the 4-sulfomuconolactone (4SL) must be fundamentally different as an elimination of the sulfonic acid substituent in analogy to the decarboxylation of 4-carboxymuconolactone to 4-oxoadipate enol-lactone is mechanistically not possible (Fig. 1). Therefore, it was previously suggested that 4-sulfomuconolactone is hydrolytically desulfonated to maleylacetate (Feigel & Knackmuss, 1993).

In the present study, the 4-sulfomuconolactone hydrolases (4SLHs) from *H. intermedia* S1 and *A. radiobacter* S2 were analysed on the enzymatic and molecular level in order to further

clarify the evolution of the modified β-ketoadipate pathway that is responsible for the conversion of various substituted sulfonated benzenes which are degraded via 4-sulfocatechol (Contzen *et al*., 1996; Schleheck *et al*., 2004; Schulz *et al*., 2000).

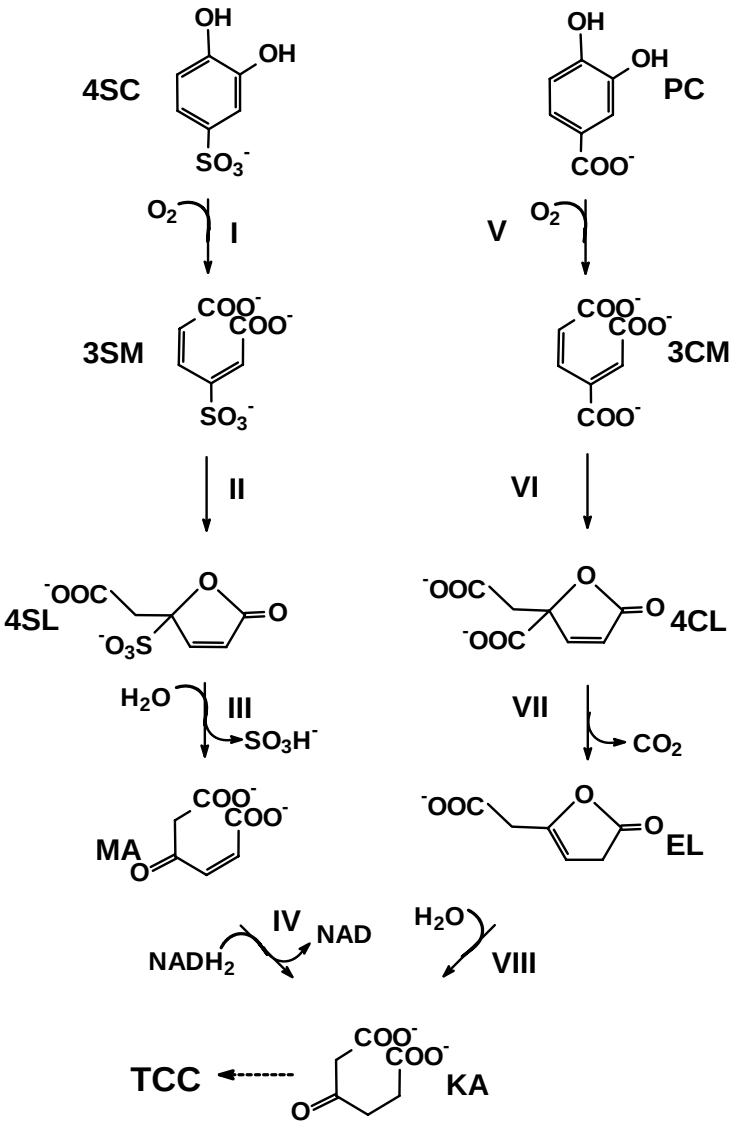

Fig. 1. **Proposed pathway for the degradation of 4-sulfocatechol and protocatechuate by *Agrobacterium radiobacter* S2 (Feigel and Knackmuss, 1993).** Key to enzymes: I, protocatechuate 3,4-dioxygenase type II; II, 3-carboxy-*cis,cis*-muconate lactonizing enzyme type II; III, 4-sulfomuconolactone hydrolase; IV, maleylacetate reductase; V, protocatechuate 3,4-dioxygenase type I; VI, 3-carboxy-*cis,cis*-muconate lactonizing enzyme type I; 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-sulfobut-2-en-4-olide); MA, maleylacetate; KA, β-ketoadipate; PC, protocatechuate; 3CM, 3-carboxy-*cis,cis*-muconate; 4CL, carboxymuconolactone; EL, β-ketoadipate enol-lactone.

METHODS

Bacterial strains and media. The isolation, characterization, and culture conditions of *Hydrogenophaga intermedia* S1 (DSMZ 5680) and *Agrobacterium radiobacter* S2 (DSMZ 5681) have been described before (Feigel & Knackmuss, 1988, 1993; Contzen *et al*., 2000). *Escherichia coli* DH5α, *E. coli* JM109, *E.coli* BL21(DE3)/pLysS and *E. coli* BL21(DE3)/pLysS Star (Invitrogen, Carlsbad, CA) were used as host strains for recombinant DNA work. *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium which was supplemented with ampicillin (100 μg/ml), if appropriate.

Plasmids and DNA manipulation techniques. The characteristics of all plasmids used are given in Tab. 1. The isolation of genomic DNA of *H. intermedia* S1 and *A. radiobacter* S2, PCR, and all DNA work were performed as described previously (Halak *et al.,* manuscript submitted).

Determination of the nucleotide sequence of the 4-sulfomuconolactone hydrolase gene from *Agrobacterium radiobacter* S2. The genes coding for the protocatechuate 3,4-dioxygenase type II and 3-carboxy-*cis,cis*-muconate lactonizing enzyme type II (ArCMLE2) which are responsible in *A. radiobacter* S2 for the conversion of 4-sulfocatechol to 4-sulfomuconolactone had been previously identified on plasmid pMCS2-2. Downstream of the gene encoding for ArCMLE2 a truncated gene encoding a putative hydrolase was identified (ORF 4; Contzen & Stolz, 2000; Halak *et al*., manuscript submitted). The missing part of ORF4 was obtained using partially inverse PCR (Pang & Knecht, 1997). Thus, finally a DNA-fragment was amplified from the genomic DNA of *A. radiobacter* using the primers MC_CHA_1 and MC_CHA_2 (Tab. 2). The amplified DNA fragment (ca 2 kb) was cloned into pBluescript II SK(+) giving pS2PDH-2. DNA-squencing of the insert demonstrated that it encoded the missing part of the gene encoding the 4-sulfomuconolactone hydrolase and a putative maleylacetate reductase gene.

Table 1: Bacterial plasmids

Plasmid	Relevant characteristics	Source or reference
pBlueskript II SK(+)	Standard cloning vector, ap^r	Alting-Mees et al., (1992)
pAC28	T7- Expression vector	Kholod & Mustelin, 2001
pSHSLHS1	4-Sulfomuconolactone hydrolase gene from *H. intermedia* S1 in pAC28	this study
pSHSLHS2	4-sulfomuconolactone hydrolase gene from *A. radiobacter* S2 in pAC28	this study
pMCS2-2	*pcaG2*, genes for a putative TRAP transport system, putative IclR regulator, *pcaB2S2* and the gene fragment for the C-terminal part of the 4-sulfomuconolactone hydrolase from *A. radiobacter* S2 in pBluescript II SK(+)	Contzen & Stolz, 2000
pS2PDH-2	C-terminal part of the gene for the 4-sulfomuconolactone hydrolase and the maleylacetate reductase from *A. radiobacter* S2 in pBluescript II SK(+)	this study
pETS2-X-II	Expression of *pcaH2G2* from *A. radiobacter* S2 under the control of the T7 promotor	Contzen *et al*., 2001
pDS15	Gene for the 2-pyrone-4,6-dicarboxylate hydrolase from *S. paucimobilis* SYK-6 under the control of the T-*7* promotor	Masai *et al.*, 1999

Sequencing of the 4-sulfomuconolactone hydrolase gene from *H. intermedia* S1. The gene encoding for the 4-sulfomuconolactone hydrolase from *H. intermedia* S1 was identified downstream of the gene encoding the 3-sulfomuconate converting 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) on an approximately 2-kb DNA fragment previously obtained by PCR using the primers pcaBS1_1930F and pcaBS1_1888R (Halak *et al.*, manuscript submitted). The DNA-fragment was completely sequenced and found to encode the carboxyterminal part of the CMLE, the 4SLH, and the aminoterminal part of a maleylacetate reductase.

Amplification and cloning of the 4-sulfomuconolactone hydrolases in *E. coli*. The genes encoding the 4-sulfomuconolactone hydrolases of *H. intermedia* S1 and *A. radiobacter* S2 were amplified from the genomic DNAs of the strains by PCR using the oligonucleotide primers PDHS1-X-N, PDHS1-X-B, PDHS2-X-N, and PDHS2-X-C (Tab. 2) using "Ready to Go" PCR beads (Amersham). This resulted in the simultaneous introduction of *NdeI* sites upstream and *BamHI* sites downstream of the genes. The amplification was started by a "touch-down" PCR (30 s 95°C for denaturation, 1 min 72°C for elongation) with 30 cycles using an annealing temperature of 65 °C which was followed by 29 cycles using an annealing temperature of 57°C The amplified products were (partially) cleaved with *Nde*I and *Bam*HI (there is a *Nde*I cleavage site in the 4-sulfomuconolactone hydrolase gene of strain S1) and cloned into pAC28 (Kholod and Mustelin, 2001) which was previously also cut with *Nde*I and *Bam*HI. This resulted in the recombinant plasmids pSHSLHS1 and pSHSLHS2, which encoded for aminoterminally His-tagged enzyme variants. The plasmids were subsequently used to transform cells of *E. coli* BL21(DE3)/pLysS.

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, Freiburg, Germany) with fluorescently labeled primers.

Sequence analysis, data base searches, and sequence comparisons were done with the Lasergene software package, version 5 (DNASTAR Inc., Madison) and the BLAST Search at NCBI (Altschul *et al.,* 1997). The alignments of the different enzyme sequences were obtained with the program CLUSTALX using the default parameters.

Table 2: Oligonucleotide primers used in the present study

Position	Primer name	Deduced primer sequence[a] (5′→3′)
C-terminal region of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1	MC_CHA_1	gatgcggtcgagcgttctg
Downstream of the 4-sulfomuconolactone hydrolase gene from *H. intermedia* S1	MC_CHA_2	cgaaagtgttgcagcgaccg
N-terminus of the 4-sulfomuconolactone hydrolase from *A.radiobacter* S2	PDHS2-X-N	aaaacatatgttacccgctgatcaagctgg
C-terminus of the 4-sulfomuconolactone hydrolase from *A.radiobacter* S2	PDHS2-X-C	aaaaggatccgttgcattgaatatccgcccc
N-terminus of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1	PDHS1-X-N	ttttcatatgtcagaacaagctgttgaagtttcgc
C-terminus of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1	PDHS1-X-B	ttggatcctcatgctcccttggcaacc

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

High pressure liquid chromatography (HPLC). The turn-over of 4-sulfocatechol, 3-sulfomuconate and 4-sulfomuconolactone was analyzed by reversed-phase HPLC (pumps model 510 equipped with a photo-diode array detector model 996 and Millenium Chromatography Manager 2.0, Waters Associates, Milford, Mass.). A reversed-phase column [250 × 4.0 mm (internal diameter), packed with 3 μm particles of Nucleosil C18] was used. The average flow rate was 1 ml/min. 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 average retention times of 4-sulfocatechol, 3-sulfomuconate, and 4-sulfomuconolactone under these chromatographic conditions were 3.7, 3.4, and 4.3 min, respectively.

The turn-over of 4SLH to maleylacetate was analysed using a different reversed-phase column [125 × 4.0 mm (internal diameter), packed with 5 μm particles of Lichrospher 100, RP8] and a solvent system consisting of 16 % (v/v) acetonitrile, 83.7 % water, and 0.3% (v/v) trifluoroacetic acid. In this system 4SL and maleylacetate had average retention times of 1.3 and 2.7 min, respectively.

Preparation of cell-free extracts. Cell suspensions in 50 mM Tris-HCl buffer, pH 8.0, were disrupted by using a French press (SLM Aminco; SLM Instruments Inc., Urbana, IL, U.S.A.) at 1.1 x 10^8 Pa. Cells and cell debris were removed by centrifugation at 100,000 g for 30 min at 4°C.

Protein estimation and enzyme assays. Protein content of cell-free extracts was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard. One unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate per minute.

The conversion of 4-sulfomuconolactone was routinely measured using a spectrophotometric assay. The cuvettes contained in a final volume of 1 ml 100 μM 4-sulfomuconolactone (synthesized enzymatically from 4-sulfocatechol, see below) and 50 mM Tris/HCl buffer (pH 8). The reactions were started by the addition of cell extracts or purified enzyme preparations. The increase in absorption due to the formation of maleylacetate was determined at 242 nm. The reaction rates were calculated by using a molar extinction coefficient for maleylacetate of ε_{242nm}= 4740 $M^{-1}cm^{-1}$ (Schlömann, 1988).

2-Pyrone-4,6-dicarboxylate hydrolase activity was determined spectrophotometrically by the method described by Masai *et al.* (1999). The cuvettes contained in a total volume of 1 ml 50 mM Tris/HCl (pH 8) and 100 μM 2-pyrone-4,6-dicarboxylate. The reactions were started by the addition of cell extracts and the decrease of absorption monitored at λ= 312 nm. Reaction rates were calculated by using a molar extinction coefficient of 6600 M^{-1} cm^{-1} (Masai *et al.*, 1999).

Purification of the His-tagged enzyme variants of the 4-sulfomuconolactone hydrolases. *E. coli* BL21(DE)pLysS(pSHSLHS1) and *E. coli* BL21(DE)pLysS(pSHSLHS2) were grown at 30°C in LB-medium plus kanamycin (50 μg/ml) to an optical density (OD_{546nm}) = 0.5 before isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added. The bacterial cultures were grown for another 5 h, the cells were then harvested by centrifugation and cell extracts prepared in Tris/HCl buffer (50 mM, pH 8.0). The cell extracts (about 120 mg of protein) were transferred to a 20 ml-FPLC chromatography column filled with "Ni-NTA Superflow" (Qiagen) and the column was washed with 1-2 column volumes of the equilibration buffer consisting of Tris/HCl (50 mM, pH 8.0), NaCl (300 mM), imidazole (20 mM), and 1 mM of 1,4,-dithio-D,L-threitol (DTT). The active enzymes were then eluted using a buffer system (pH 8.0) consisting of Tris/HCl (50 mM), NaCl (300 mM), DTT (1 mM), and 100 mM imidazole. Fractions (5 ml each) were collected and the fractions showing enzymatic activity (usually the third and fourth fraction) used for the enzymatic tests.

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 a "high molecular weight" (HMW) calibration kit (Amersham Biosciences).

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 (Biorad).

Determination of the metal content of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1. The purified enzyme (about 100 μg protein in 300 μl 50 mM Tris/HCl, pH 8.0) was dialysed twice at 4°C for 36 h against 4 l of 50 mM Tris/HCl (pH 8.0). The extinction coefficient of the protein was calculated from the known amino acid sequence as 42,420 M^{-1} cm^{-1} (Gill & van Hippel, 1989; http://www.basic.northwestern.edu). As the sample demonstrated an extinction at 280 nm of 0.26, the protein content of the dialysed protein solution was calculated as 6 μM. The metal content of the sample was analysed by plasma mass-spectroscopy (ICP-MS) (Spurenanalytisches Laboratorium Dr. Baumann,

Maxhütte-Haidhof, Germany). The sample contained 0.197 μg/ml Zn, 0.079 μg/ml Mg, 0.052 μg/ml Ni, 0.038 μg/ml Fe, 0.003 μg/ml Mn, and 0.001 μg/ml Co.

Spectrophotometric quantitation of sulfite. The enzymatic release of sulfite from 4-sulfomuconolactone was quantified by using Ellman´s reagent [5,5´-dithiobis(2-nitrobenzoic acid)] as described by Johnston *et al.* (1975).

Enzymatic production of 4-sulfomuconolactone. The 4-sulfomuconolactone for the enzyme assays was prepared by two subsequent enzymatic reactions: 4-Sulfocatechol (4 mM in 1 ml 50 mM Tris-HCl, pH 8.0) was converted to 3-sulfomuconate using a cell extract from *E. coli* BL21(DE3)(plysS)(pETS2-X-II) (4.5 mg of protein), which heterologously expressed the protocatechuate 3,4-dioxygenase type II (P34OII) from *A. radiobacter* S2 (Contzen & Stolz, 2000; Halak *et al*., manuscript submiteda). The reaction mixtures were incubated in an Eppendorf shaker at 1400 rpm and 30° C and the reaction was monitored by HPLC until substrate depletion (after approximately 45 min). Then, 50 μl (0.5 mg protein/ml) of a purified preparation of the 3-sulfomuconate converting 3-carboxy-*cis,cis*-muconate lactonzing enzyme from *A. radiobacter* (ArCMLE1; Halak *et al*., manuscript submitted) was added. The reaction mixtures were shaken and the reaction was further analysed by HPLC until the complete conversion of the 3-sulfomuconate was achieved (usually after 25- 30 min). The proteins were removed by centrifugation in an ultrafiltration unit (Vivaspin 2ml Concentrator, 10000 MWCO, PES membrane) and the filtrates used for the enzyme assays.

In situ ^{1}H NMR analysis of the transformation of 4-sulfocatechol. 4-Sulfocatechol was dissolved in 4 ml of 50 mM Tris-HCl-buffer (pH 8.0) to give a final concentration of 4 mM. An aliquot of this solution was supplemented with 20% (v/v) D_2O and transferred to the NMR sample tube (0.7 ml). The one-dimensional ^{1}H NMR spectra were recorded at 300 K on an AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten, Germany) locked to the deuterium resonance of D_2O in the solution. Spectra were recorded by using the standard Bruker 1D NOESY suppression sequence with 280 scans, each with a 1.8-s acquisition time and 1.3-s relaxation delay. The center of the suppressed water signal was used as an internal reference (4.80 ppm).

4SC (4 mM in 1 ml 50 mM Tris-HCl, pH 8.0) was converted by the addition of a cell extract (26 mg of protein) from *E. coli* BL21(D3)(plysS)(pETS2-X-II) (Contzen & Stolz, 2000) which expressed the 4SC converting protocatechuate 3,4-dioxygenase type II. The reaction was analysed in parallel using HPLC and NMR. The HPLC analysis [solvent system: 1 % (v/v) methanol, 98.9 % water, and 0.1% (v/v) H_3PO_4] demonstrated that the addition of the enzyme preparation resulted within 30 min in the complete conversion of 4SC (R_t= 3.7 min) to a product with a retention time of R_t= 3.4 min. A part of the solutions containing the substrate or the product were simultaneously analysed by NMR.

In the following step, a diluted sample (1:1 v/v; 1 ml) of the product solution (containing about 2 mM 3-sulfomuconate) was incubated with 50 µl (0.1 mg/ml) of the purified 3-sulfomuconate converting CMLE from *H. intermedia* (HiCMLE2). [The His-tagged HiCMLE2 was purified by affinity chromatography as described previously by Halak *et al.* (manuscript submitted) and the imidazole, which inhibited the enzyme, removed by ultrafiltration (Vivaspin 20, Sartorius) and subsequent dilution of the concentrated protein solution in 50 mM Tris/HCl, pH 8.0]. The reaction mixture was incubated on a laboratory shaker, aliquots were taken after 10, 30, 60, 90, and 120 min and analysed by HPLC. This demonstrated that 3-sulfomuconate was completely converted after 60 min. The sample was then analysed by NMR (see results section).

The solution containing the 4-sulfomuconolactone (1 ml, 2 mM) was incubated with the purified 4-sulfomuconolactone hydrolase from *H. intermedia* S1 (50 µl, 0.5 mg/ml). The HPLC analysis demonstrated that the substrate was completely converted within 60 min. The product formed was identified by NMR in comparison to a previously produced sample (Pieper *et al*., 2002) as maleylacetate.

Chemicals: 4-Sulfocatechol was synthesized according to the procedure described by Quilico (1927).

Maleylacetate was prepared from 4-carboxymethylenebut-2-en-olide (*cis*-dienelactone) by alkaline hydrolysis (Kaschabek & Reineke, 1995). The *cis*-dienelactone was dissolved in water (2 mM, 10 ml) and 50 µl of 1 M NaOH added. The formation of maleylacetate was analysed spectrophotometrically at 242 nm until no further increase in absorbance was observed.

cis-Dienelactone and 2-pyrone-4,6-dicarboxylate were kindly provided by Prof. M. Schlömann (TU Freiberg, Germany) and Dr. E. Masai (Nagaoka University of Technology, Japan). All other chemicals used were obtained from Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), and Sigma (Neu-Ulm, Germany).

Nucleotide sequence accession number. The nucleotide sequences of the sulfomuconolactone hydrolases will appear in the GenBank nucleotide sequence data base under the accession numbers DQ813261 and DQ813262, respectively.

RESULTS

Identification, cloning and sequencing of the genes for the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 and *A. radiobacter* S2. Previously, the genes encoding two 3-carboxy-*cis,cis*-muconate lactonizing enzymes were identified in the genomes of *H. intermedia* S1 and *A. radiobacter* S2. These genes were found in both strains in close proximity to the genes coding for protocatechuate 3,4-dioxygenases. Furthermore, it was shown that the encoded proteins were able to convert 4-sulfocatechol (P34OII) and 3-sulfomuconate (CMLE2s). Therefore, it was postulated that in both strains the genes encoding the P34OIIs (*pcaH2G2*) and CMLEs (*pcaB2*) were part of genetic structures which were responsible for the degradation of 4-sulfocatechol. In the postulated degradative pathway (Fig. 1) the product of the cycloisomerization reaction of 3-sulfomuconate is a sulfonated lactone which should undergo a previously undescribed enzymatic hydrolytic desulfonation to maleylacetate. In order to search for the encoding gene, the genomic regions downstream of the *pcaB2* genes were sequenced in *H. intermedia* S1 and *A. radiobacter* S2. Thus, in both strains downstream of the respective *pcaB2* genes two rather similar genes were identified. The genes encoded proteins of 303 (strain S1) or 299 (strain S2) amino acids (Fig. 2), which showed 57 % sequence identity to each other.

Sequence comparisons demonstrated for both enzymes the highest degree of sequence identity (57- 68 %) to a recently sequenced putative dicarboxylic acid hydrolase from *Novosphingobium subarcticum* SA1 (NCBI number AAW29742). Presumably, this enzyme belongs to the sulfanilate/4-sulfocatechol degradation gene cluster in this organism (NCBI number AY700015). A comparison of the putative 4-sulfomuconolactone hydrolases with sequences deposited at the NCBI data base for which experimentally an enzymatic activity has been determined demonstrated the highest degree of sequence identity (30- 32%) to the 2-pyrone-4,6-dicarboxylate hydrolase (PDCH) from *Sphingomonas paucimobilis* SYK-6. The PDCHs take part in the extradiol *meta*-cleavage pathway of protocatechuate (Masai *et al*., 1999; Maruyama *et al.*, 1994). A comparison of the structural features of the substrates of the PDCHs (2-pyrone-4,6-dicarboxylate) and the 4-sulfomuconolactone hydrolases (4-carboxymethylen-4-sulfo-but-2-enolide) showed that both substrates resembled each other in their lactone structure and the presence and relative position of negatively charged groups (Fig. 3). This indicated that the two genes cloned from *H. intermedia* S1 and *A. radiobacter* S2 presumably encoded for the 4-sulfomuconolactone hydrolase activities in the organisms investigated.

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SLH H.intermedia        MSEQAVEVSPKCLGPQHHINPLRFVMPPGSWDTHFHVFGPTTKYPYSETRKYTPPDSPFE 60
SLH A.radiobacter       MLPADQAGIPPCQGPRARSAPISFAIPKGAWDTHLHVFGPTAVFPYAEKRPYTPPDSPLE 60
PDH P.putida            --------MKSCLPPDPSPSKPQQSLPAGSWDAHCHVFGPAKDFPYSEDRSYTPPDASFS 52
PDH S.paucimobilis      -----MTNDERILSWNETPSKPRYTPPPGAIDAHCHVFGPMAQFPFSPKAKYLPRDAGPD 55
                                                * *: *:* *****   :*::    * * *:  .

SLH H.intermedia        EYVKLMLALGIERGVCVHPNIHGPDNSVTLDAVERSEGRFLAIVKIAPDVTLPQLKEMKK 120
SLH A.radiobacter       DYLALMERLGIERGVCVHPNVHGIDNSVTIDAVERSDRRLLGIIKPHRVMTFTELRDLKT 120
PDH P.putida            QLLDLHDHLGFDRGVIVQASCHGTDNTAMLDAIGRSAGRYRGVAIISGTETDRQLAEMDA 112
PDH S.paucimobilis      MLFALRDHLGFARNVIVQASCHGTDNAATLDAIARAQGKARGIAVVDPAIDEAELAALHE 115
                          . *   **: *.* *:.. ** **:. :**: *:  :  .:          :*  :.

SLH H.intermedia        KGACGVRFAFNPEHGSGELDTALFDRVVQWCGELDWCVNLHFASNAIHSLAERLSQLTIP 180
SLH A.radiobacter       RGVRGVRFAFNPQHGSGALDTELFERMHGWCRELDWCINMHFAPDALEGLCDLIAGAETP 180
PDH P.putida            GGVRGVRFNFVAHLG-GAPDLEVFDRALERIEQFGWHVVLHLDAQDIVTYADRLERIKVP 171
PDH S.paucimobilis      GGMRGIRFNFLKRLVDDAPKDKFLEVAGRLP--AGWHVVIYFEADILEELRPFMDAIPVP 173
                         *  *:** *  .  ..  .  .::       . .* : ::: .: :      :     *

SLH H.intermedia        TLIDHFGRVHPTKGVDQPDFKTLVDLMR-LPHMWVKLTGADRISRNSPSYQDVVPLARTL 239
SLH A.radiobacter       IIIDHFGRVETAAGVNQLPFKILRDLAT-LDHVWIKLTGADRISHSGVPYDDVVPFAHAL 239
PDH P.putida            FVIDHMGRVKAQDGLDQAPFRALVELME-NPLAWVKVCGAERVSAGRKPFDDAIPFAMAL 230
PDH S.paucimobilis      IVIDHMGRPDVRQGPDGADMKAFRRLLDSREDIWFKATCPDRLDPAGPPWDDFARSVAPL 233
                         :***:** .   * :   :: :  *       *.*   .:*:.    .::*    . .*

SLH H.intermedia        VDVAPDRVIWGTDWPHSGYFDVKRMPNDGDLTNLLLDFAPSEEQRRRILVDNPSRLFGQV 299
SLH A.radiobacter       SEIAPDRLLWGSDWPHSGYFDPKRMPDDGDLLNLVARFAPDVALRHKILVDNPARLFGVI 299
PDH P.putida            IETAPERVLWGTDWPHPNIS--KDMPNDGGLVDLMHRFCPDDSTRRKLLIENPLKLYGR- 287
PDH S.paucimobilis      VADYADRVIWGTDWPHPNMQD--AIPDDGLVVDMIPRIAPTPELQHKMLVTNPMRLYWSE 291
                            .:*::**:****..  . . :*:** : :::  :.*    ::::*: ** :*:

SLH H.intermedia        AKGA 303
SLH A.radiobacter       ----
PDH P.putida            ----
PDH S.paucimobilis      EM-- 293
                        .:
```

Fig. 2. Sequence alignment of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1 and *A. radiobacter* S2 and the 2-pyrone-4,6-dicarboxylate hydrolases from *Sphingomonas paucimobilis* SYK-6 and *Pseudomonas ochraceae* NGJ1.

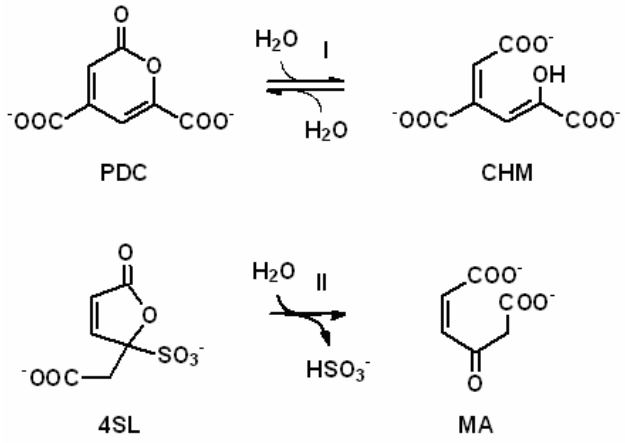

Fig. 3. Comparison of the proposed reaction catalysed by 2-pyrone-4,6-dicarboxylate hydrolases and 4-sulfomuconolactone hydrolases.

Evidence for the structural coupling of the genes encoding 4-sulfomuconolactone hydrolases and maleylacetate reductases. A BLAST search using the nucleotide sequences from *H. intermedia* S1 downstream of the 4-sulfomuconolactone hydrolase gene identified an ORF with significant homology to genes encoding maleylacetate reductases. Thus the ORF finder of the BLAST program suggested a truncated ORF encoding 244 aa of a protein which showed 63% sequence identity with the amino acids 29-217 of a putative maleylacetate reductase from the the putative 4-sulfocatechol operon of *Novosphingobium subarcticum* (NCBI No AAW29743.1) and 61% sequence identity with the amino acids 23-217 of a presumed malelylacetate reductase from the 2,4D degrading *Burkholderia cepacia* 2a (NCBI No AAK81685.1).

Also in *A. radiobacter* S2 downstream of the 4-sulfomuconolactone hydrolase gene a gene encoding a putative maleylacetate reductase was identified (on plasmid pS2PDH-2). The deduced gene product with a length of 351 aa showed again the highest degree of sequence identity (= 63%) with the sequence deposited for *Novosphingobium subarcticum* (NCBI No AAW29743.1).

Thus in both strains a conserved gene order encoding a 3-carboxy-*cis,cis*-muconate lactonizing enzyme, a putative 4-sulfomuconolactone hydrolase and a putative maleylacetate reductase were found (Fig. 4). Maleylacetate reductases are involved in the proposed degradative pathway of 4-sulfocatechol (Fig. 1). This suggested that in both strains an operon structure exists which might be conserved in the degradative pathway of substituted benzenesulfonates.

Functional expression of the 4-sulfomuconolactone hydrolases in *E. coli*. The ORFs encoding for the 4-sulfomuconolactone hydrolases were amplified by PCR from the genomic DNAs of strains S1 and S2 using the primers PDHS1-X-N, PDHS1-X-B, PDHS2-X-N, and PDHS2-X-C (Tab. 2) and cloned into the expression vector pAC28 (see materials and methods). This resulted in the formation of plasmids pSHSLHS1 and pSHSLHS2 which encoded for the His-tagged variants of the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 and *A. radiobacter* S2, respectively. The encoded enzymes were functionally expressed in *E. coli* BL21(DE3)/pLysS using IPTG (see materials and methods) and cell extracts analysed by SDS gel electrophoresis. Thus, in both clones the induction of a protein with a subunit mass of about 35 kDa was observed.

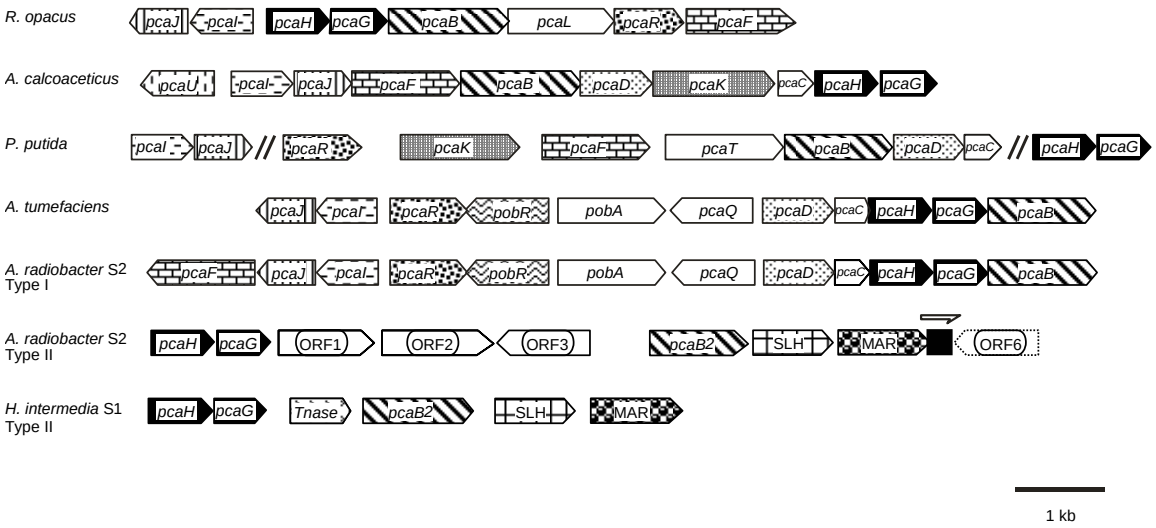

Fig. 4. Structures of the gene clusters for the catabolism of protocatechuate and 4-sulfocatechol from *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 in comparison to the protocatechuate gene clusters from *Rhodococcus opacus, Acinetobacter calcoaceticus, Pseudomonas putida,* and *Agrobacterium tumefaciens* (Eulberg *et al.*, 1998; Parke, 1995, 1997).

Identification of maleylacetate as reaction product. The enzyme variants were purified on nickel-agarose columns by IMAC (immobilized metal ion affinity chromatography). The purified proteins were incubated with 4-sulfomuconolactone which was enzymatically prepared from 4-sulfocatechol by using protocatechuate 3,4-dioxygenase and sulfomuconate lactonizing enzymes (see materials and methods section). The spectrophotometrical analysis of the reaction using overlay spectra clearly demonstrated that the 4-sulfomuconolactone was converted by the enzymes to a product which showed an increased absorbance around 240 nm (Fig. 5). This indicated that indeed maleylacetate was the reaction product, because for this compound at neutral pH-values an absorption maximum of 245 nm has been described (Chapman & Ribbons, 1976). Further evidence for the identity of the product of the 4-sulfomuconolactone hydrolase reaction with maleylacetate was obtained by acidification of the reaction mixture to pH 2 (which resulted in the disappearance of the absorption maximum at 240 nm) and subsequent reneutralization (which restored the absorbance at 240 nm) (Chapman & Ribbons, 1976).

The presence of two isosbestic points in the overlay-spectra suggested that the substrate was stoichiometrically and directly converted into the product. Therefore, a defined concentration of 4-sulfocatechol (0.1 mM) was enzymatically converted to the 4-sulfomuconolactone and this solution incubated with the purified 4-sulfomuconolactone hydrolase from *E. coli*

BL21(DE)pLysS(pSHSLHS1). Thus it was found that the amount of 4-sulfomuconolactone which was enzymatically produced from 0.1 mM 4-sulfocatechol resulted after the addition of the purified 4-sulfomuconolactone hydrolase in an increase in absorbance at 242 nm (due to the formation of the product) of 0.48. This value nicely correlated with the reported molar extinction coefficient of maleylacetate of 4740 M^{-1} cm^{-1} reported by Schlömann (1988) indicating that 4-sulfomuconolactone was indeed stoichiometrically converted to maleylacetate.

The formation of maleylacetate was further substantiated by HPLC analysis of the reaction. Thus, 4-sulfomuconolactone (1 mM in 50 mM Tris/HCl, pH 8.0) was incubated in 1 ml for 20 min at room temperature with 50 µg of the purified 4-sulfomuconolactone hydrolase from *H. intermedia* S1 and the reaction analysed by HPLC [solvent system:16 % (v/v) acetonitrile, 83.7 % water, and 0.3% (v/v) trifluoroacetic acid]. This resulted in the disappearance of the signal for 4SL (R_t= 1.3 min) and the formation of a new signal with a retention time of 2.7 min. The newly formed product was identified in comparison to an authentic standard according to its retention time and in-situ spectrum as maleylacetate. These experiments demonstrated that the 4-sulfomuconolactone was indeed converted by the 4-sulfomuconolactone hydrolase to maleylacetate.

The 4-sulfomuconolactone hydrolysing activities of the recombinant *E. coli* strains were calculated from the spectrophotometric test by using the increase in absorbance at 242 nm and a molar extinction coefficient for maleylacetate of 4740 $M^{-1}\times cm^{-1}$. Thus, in *E. coli* JM109(pPDHS1) and *E. coli* JM109(pPDHS2) SLH activities of 0.25 and 0.05 U/mg of protein, respectively, were found.

Identification of sulfite as reaction product. The hydrolytic desulfonation of 4-sulfomuconolactone should result in the formation of maleylacetate plus sulfite. Therefore, different concentrations of 4-sulfomuconolactone (0.02- 0.08 mM in 50 mM Tris/HCl-buffer, pH 8.0) were incubated for 10 min with the purified 4-sulfomuconolactone hydrolase from *H. intermedia* S1 (10 µl, c= 0.5 mg/ml) and the amount of sulfite formed determined using Ellman´s reagent (Johnston *et al.*, 1975). Thus, in all experiments almost stoichiometrical amounts (R^2= 0.99) of sulfite were formed from the 4-sulfomuconolactone. In control experiments with the respective 4-sulfomuconolactone concentrations without added enzyme

no increase in absorbance at 415 nm due to the added 4-sulfomuconolactone solution was observed.

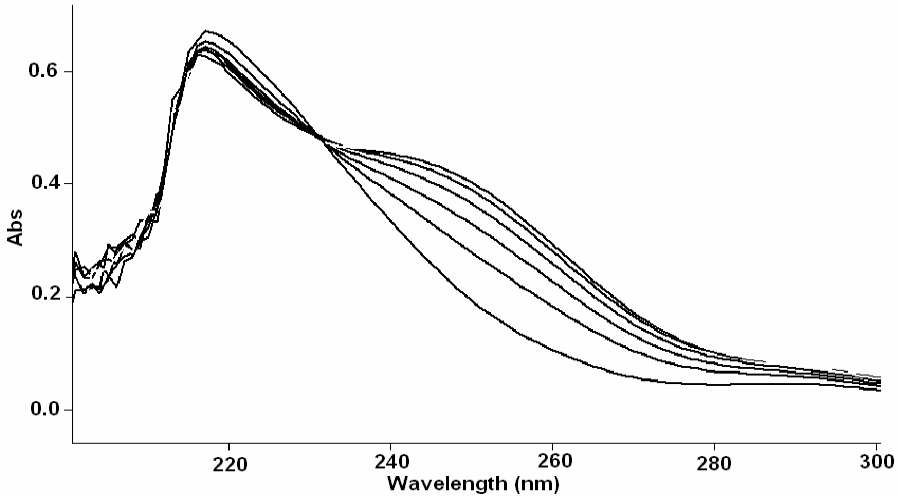

Fig. 5. Spectrophotometric analysis of the conversion of 4-sulfomuconolactone by the purified 4-sulfomuconolactone hydrolase from *H. intermedia* S1. The reaction mixture contained in a final volume of 1 ml 50 mM Tris-HCl (pH 8.0), 100 µM 4-sulfomuconolactone, and 5 µl of a purified preparation of the 4-sulfomuconolactone hydrolase from *H. intermedia* (C_{prot} = 0.5 mg/ml). The overlay spectra were recorded every min against a reference cuvette containing the same ingredients but no enzyme.

Enzymatic characterization of the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 and *A. radiobacter* S2. The His-tagged enzymes were purified by IMAC and the molecular masses of the holoenzymes determined by gel filtration. Thus, for the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 and *A. radiobacter* S2 molecular weights of 34700 and 32900, respectively, were determined. This suggested that both enzymes have monomeric structures as previously found for the PDCH from *S. paucimobilis* SYK-6 (Masai *et al*., 1999).

The basic catalytic constants were determined for both enzymes using different concentrations of 4-sulfomuconolactone in 50 mM Tris-HCl (pH 8.0). Thus, it was found that the enzyme from strain S1 showed significanly higher v_{max} and k_M-values, but that the catalytic constants (k_{cat}/k_M) of both enzymes were rather similar (Tab. 3).

Table 3: Kinetic data for the conversion of 4-sulfomuconolactone by the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 and *A. radiobacter* S2

Enzyme	Source	MW per subunit (kDa)	K_M (mM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
HiSLH	*H.intermedia* S1	34.7	1.9 ± 0.5	79.0 ± 18.6	2693	1417
ArSLH	*A. radiobacter* S2	32.9	0.34 ± 0.14	12.6 ± 3.3	419	1233

The data were calculated by non-linear regression using the Prism 4 software (GraphPad Software, San Diego, CA).

Indications for the presence of Zn^{2+}-ions in the active center of the 4-sulfomuconolactone hydrolases. Sequence comparisons at the NCBI using the "Conserved Domain Search" program suggested some sequence similarities in the carboxyterminal parts of the 4-sulfomuconolactone hydrolases, PDCHs and a group of enzymes generally summarized as cyclic amidases, such as D-hydantoinases and dihydropyrimidases. These cyclic amidases usually contain divalent cations, such as Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+} in their active centers (Kim & Kim, 1998). Therefore, the metal content of a sample of the purified His-tagged variant of the 4-sulfomuconolactone hydrolases from *H. intermedia* S1 was determined by plasma mass-spectroscopy (ICP-MS). The sample contained a relatively high amount of Zn^{2+}, which was significantly higher than the concentration of Ni^{2+} (which was used during the purification of the enzyme). The calculation of the Zn^{2+}-content and the protein concentration in the sample suggested that about 0.6 mol of Zn^{2+} were present per mol of enzyme (see materials and methods).

4-Sulfomuconolactone hydrolases do not convert 2-pyrone-4,6-dicarboxylate. The sequence comparisons and the structural similarities of the substrates suggested the possibility of some cross reactivities among the established PDCHs and the 4-sulfomuconolactone hydrolases. Therefore, PDC (0.1 mM in 1 ml 50 mM Tris/HCl, pH 8.0) was incubated with the purified 4SLH from *H. intermedia* S1 (5 µl, 4.5 mg of protein, 0.25 U/mg SLH activity) and the reaction analysed spectrophotometrically. Under these conditions no changes in absorbance at 200- 400 nm was observed. Because PDC show a pronounced absorbance maximum at λ= 312 nm, this indicated that 4-sulfomuconolactone hydrolases do not convert PDC. In addition, a cell extract from *E. coli* JM109(pDS15) which heterologously expressed the PDCH from *S. paucimobilis* SYK-9 (Masai *et al.,* 1999) was incubated with 4-sulfomuconolactone and PDC and the reactions analysed spectrophotometrically. These extracts converted PDC (0.3 U/mg of protein), but did convert 4-sulfomuconolactone. Thus, 4-sulfomuconolactone hydrolases and PDCHs are distinct enzyme activities.

NMR analysis of the complete 4SC pathway and confirmation of maleylacetate as first desulfonated intermediate. The proposed metabolic pathway of 4-sulfocatechol was finally confirmed by ^{1}H NMR analysis of the in-situ formed metabolites. Thus, 4-sulfocatechol and its metabolites were subsequently incubated with a cell extract from *E. coli*

BL21(DE3)(plysS)(pETS2-X-II) expressing protocatechuate 3,4-dioxygenase type II, a purified preparation of the 3-carboxy-*cis,cis*-muconate lactonizing enzyme (Halak *et al.*, manuscript submitted) and the 4-sulfomuconolactone hydrolase from *H. intermedia* and the metabolic reactions monitored by ^{1}H NMR analysis.

The substrate showed the presence of three aromatic protons resonating at 6.99 (d, J=8.2 Hz), 7.27 (dd, J=8.2 and >2 Hz), and 7.31 ppm (d, J < 2Hz) (Fig. 6) in accordance with the 4-sulfocatechol structure (Feigel and Knackmuss, 1988). Turnover by the protocatechuate dioxygenase type II resulted in the formation of 3-sulfomuconate as confirmed by the ^{1}H NMR spectrum. Three olefinic protons could be observed, which gave signals at δ = 6.13 (H5), 6.40 (H4) and 6.62 (H2) ppm, respectively. The large coupling constant between H4 and H5 indicates that they are located in an open-chain configuration as present in muconates. Addition of HiCMLE2 resulted in the formation of a product with NMR characteristics previously reported for 4-sulfomuconolactone (Feigel and Knackmuss 1993). Two olefinic protons resonate at δ = 6.43 and 7.87 ppm, respectively, and the small coupling constant of 5.6 Hz indicates the presence of the olefinic protons in a closed five-membered ring system. Two protons of a methylene group showed signals at δ = 3.21 and 3.27 ppm respectively. The signals were split into doublets due to a geminal coupling of 14.3 Hz. The 4-sulfomuconolactone hydrolase converted 4-sulfomuconolactone into a single product with characteristics previously reported for maleylacetate (Pieper *et al*., 2002). Two olefinic protons (δ = 6.34 and 6.47 ppm) were evidently present in an open-chain formation as evidenced by the coupling constant of 12 Hz, and two methylene protons were shown to resonate at δ = 3. 56 ppm.

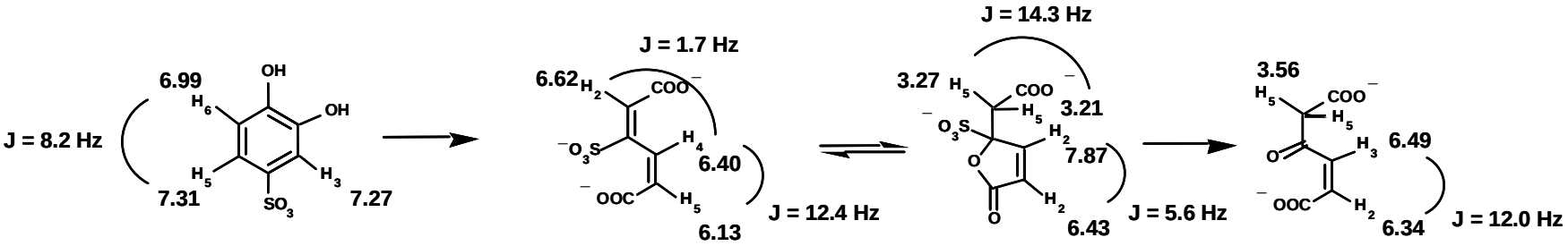

Fig.6. Proposed explanations for the ^{1}H NMR signals obtained for 4-sulfocatechol and its transformation products.

DISCUSSION

The present study represents the final part of our investigation about the enyzmes and genes involved in the degradation of 4-sulfocatechol (Contzen & Stolz, 2000; Contzen *et al*., 2001; Halak *et al*., manuscript submitted). This study was undertaken because 4-sulfocatechol has been identified as a central intermediate in the microbial degradation of various substituted benzenesulfonates, such as sulfanilate, 1,3-benzenedisulfonate, and 4-sulfophenol (Contzen *et al*., 1996; Cook *et al*., 1999). Furthermore, 4-sulfocatechol occurs to be the first common intermediate in the degradation of the different isomers of linear alkylbenzenesulfonates (LAS) which are produced world-wide in the million-ton scale per year (Schulz *et al*., 2000; Schleheck *et al*., 2004). The importance of this degradative pathway for the environment is stressed by the statement that LAS are probably the quantitatively most important class of xenobiotic compounds which are deliberately released by mankind into the environment (Berth and Jeschke, 1989; Jimenez *et al.,* 1991).

The present study clearly demonstrated that the 4-sulfomuconolactone hydrolases are evolutionary not related to the 4-oxoadipate enol-lactone hydrolases participating in the *ortho*-cleavage pathways of catechol and protocatechuate, but are much more closely related (although distinct from) to the 2-pyrone-4,6-dicarboxylate hydrolases from the extradiol cleavage pathway of protocatechuate. It was previously suggested by Masai *et al.* (1999) that the PDCH from *S. paucimobilis* SYK-6 might contain a catalytically active cysteine residue in its active center, because the enzyme was sensitive against thiol reagents such as Ellman´s reagent and N-ethylmaleimide. Furthermore, the authors proposed from the surrounding amino acids a specific cysteine residue that most probably could fulfil this function (Cys30 in Fig. 2). It was therefore surprising that in our sequence comparisons of the 4-sulfomuconolactone hydrolases and PDCHs this cysteine residue was not conserved but replaced by phenylalanine or leucine residues in the 4-sulfomuconolactone hydrolases. A subsequent sequence analysis using the "Conserved Domain Search" at the NCBI data base then indicated some homologies between PDCHs, 4sulfomuconolactone hydrolases and the so-called cyclic amidases, which encompass *e.g.* D-hydantoinases, dihydropyrimidases, allantoinases, and dihydroorotases. All these enzymes hydrolyse cyclic amides and contain divalent metal ions, such as Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+}, which presumably are bound to the enzymes by some highly conserved histidine residues (Kim & Kim, 1998). Some of these conserved histidine residues are also present in the PDCHs and 4-sulfomuconolactone

hydrolases. Furthermore, the analysis of the metal content of the 4-sulfomuconolactone hydrolase from *H. intermedia* S1 demonstrated that significant amounts of Zn^{2+}-ions are present in the enzyme. This might indicate that these enzymes also belong to the group of enzymes currently known as cyclic amidases. This also seems reasonable because of the structural resemblance of the converted substrates, which all are 5- or 6-membered ring systems containing 1-2 amide or ester groups.

The inability of the 4-sulfomuconolactone hydrolases and the PDCH to convert the respective substrates of the other group of hydrolases clearly demonstrated that these enzymes are indeed different. This was also reflected by the presence of certain conserved amino acid residues within the sequences of the 4-sulfomuconolactone hydrolases which differed from the homologous positions in the PDCHs. Furthermore, also the positions of the respective genes within the genomes were different. Thus, the genes for both 4-sulfomuconolactone hydrolases were found (probably within operon structures) between the genes encoding (sulfomuconate converting) CMLEs and genes presumably encoding maleylacetate reductases. In contrast, the genes coding for the enzymatically characterized PDCHs from *S. paucimobilis* SYK-6 and *Pseudomonas ochraceae* NGJ1 (and also the genes presumably encoding PDCHs in *Sphingomonas* sp. LB126, *Comamonas testosteroni* BR6020, and *Arthrobacter keyseri*) were all found in operon structures which encoded all relevant enzymes of the protocatechuate *meta*-cleavage pathway (Maruyama *et al*., 2004), This extradiol degradative pathway does not include enzymes with any significant homology to CMLEs or maleylacetate reductases. Thus it should be possible in the course of sequencing projects to differentiate between the genes encocding PDCHs and 4-sulfomuconolactone hydrolases not only from the sequences of the individual genes but also by the genomic context.

The complete study about the degradation of 4-sulfocatechol demonstrated that 4-sulfocatechol and 3-sulfomuconate were converted by enzymes which clearly originate from the protocatechuate branch of the 3-oxoadipate pathway (Contzen & Stolz, 2000; Contzen *et al*., 2001; Halak *et al*., manuscript submitted). In contrast, different evolutionary origins could be shown for the lactones hydrolysing enzymes which convert 4-carboxymethylene-4-carboxy-but-2-en-olide ("4-carboxymuconolactone") in the protocatechuate pathway or 4-carboxymethylene-4-sulfo-but-2-en-olide ("4-sulfomuconolactone") in the sulfocatechol pathway. This clearly resembles the situation observed for the degradation of

chlorocatechols, which in most cases are degraded via a modified version of the catechol branch of the 3-oxoadipate pathway. Also in this metabolic pathway it was found that the chlorocatechols and chloromuconates converting enzymes were clearly homologous to the enzymes involved in the metabolism of the naturally occuring substrate benzoate. In contrast, also in the catechol and chlorocatechol pathways different types of hydrolases are necessary for the ring-opening of the intermediately formed lactone ring structures which do not show any evolutionary relationship with each other (Schlömann, 1994). Thus, it appears that nature has found in both branches of the 3-oxoadipate pathway rather similar strategies for the evolution of metabolic pathways which effort the release of substituents as anions. An interesting variation of this theme in the 4-sulfocatechol pathway is the observed pronounced sequence similarity between the 4-sulfomuconolactone hydrolases and the 2-pyrone-4,6-dicarboxylate hydrolases. This suggested a rather unique evolutionary connection between the intradiol- and the extradiol pathways for the degradation of aromatic compounds.

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