**CHARACTERIZATION OF TETRACHLOROHYDROQUINONE REDUCTIVE
DEHALOGENASE FROM *SPHINGOMONAS* sp. UG30**

A Thesis

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The Faculty of Graduate Studies

of

The University of Guelph

by

MARC B. HABASH

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ABSTRACT

CHARACTERIZATION OF TETRACHLOROHYDROQUINONE REDUCTIVE DEHALOGENASE FROM *SPHINGOMONAS* sp. UG30

Marc B. Habash
University of Guelph, 2003

Advisors:
Dr. J.T. Trevors
Dr. H. Lee

This thesis is an investigation of the tetrachlorohydroquinone reductive dehalogenase (PcpC) from *Sphingomonas* sp. UG30. PcpC is the second of five enzymes that catalyze the initial degradation of pentachlorophenol in several bacterial strains. The characterization of the wildtype UG30 PcpC revealed a 94% primary sequence identity with the well-studied PcpC from *Sphingobium chlorophenolicum* ATCC 39723. The UG30 PcpC had temperature and pH optima of 50°C and 8.7, respectively. Reduced glutathione and a reducing agent (dithiothreitol) were necessary for activity. The *S. chlorophenolicum* PcpC is susceptible to severe inhibition by its substrate tetrachlorohydroquinone, necessitating the use of a mutant enzyme for kinetic studies. In contrast, the UG30 PcpC is not inhibited by tetrachlorohydroquinone, allowing kinetic studies of the wildtype UG30 PcpC. These studies have indicated an enhanced ability to dehalogenate tetrachlorohydroquinone to dichlorohydroquinone relative to the *S. chlorophenolicum* PcpC.

An examination of N-terminal Tyr, Ser, and Cys residues by site-directed mutagenesis indicated that Ser12 and Cys14 are essential for PcpC activity, while Tyr7 and Tyr9 do not appreciably affect activity. Ser15 was also involved in the activity of

PcpC, though its role appears to be more structural in nature. An interesting observation of the Cys14Ala mutant of the UG30 PcpC was a reduced reaction rate, while the dehalogenation of tetrachlorohydroquinone to dichlorohydroquinone proceeded as in the wildtype enzyme. In contrast, the *S. chlorophenolicum* PcpC Cys14 mutant was found to have no change in reaction rate relative to the wildtype enzyme, however, only glutathione conjugated dead-end metabolites were formed. Thus, the role of the N-terminal Cys residue may reflect different reaction mechanisms in these two PcpC enzymes.

Chemical modification and site-directed mutagenesis of His and Lys residues in the UG30 PcpC suggested that His154 and Lys114 are involved in the orientation of glutathione in the active site of PcpC. Models of the PcpCs were also generated using the 3D-PSSM fold recognition server.

This research has elucidated important aspects of the structure-function of the UG30 PcpC as a member of the glutathione transferases and as a reductive dehalogenase.

Dedicated to Christie,

This is not my achievement. This is our achievement.

Thank you for your love, patience, and support.

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LIST OF ABBREVIATIONS

Full Name	Abbreviation
3-Chlorobenzoate	3-CB
Adenosine triphosphate	ATP
Chloroethane	CEA
Chlorohydroquinone	CHQ
cis-1,2-Dichloroethylene	cis DCE
Chloro-hydroxy-benzoate	Cl-OHB
Chloro-hydroxy-phenylacetate	Cl-OHPA
Chlorophenol	CP
Dichlorobenzoyl CoA	DCB CoA
Dichlorohydroquinone	DCHQ
Dichloromethane	DCM
Dichlorophenol	DCP
Dithiothreitol	DTT
Fumarylacetoacetone	FAA
Iron Sulfur	FeS
Reduced glutathione	GSH
Oxidized glutathione	GSSG
Glutathione transferase	GST
Hydroquinone	HQ
Maleylacetone	MA
Maleylacetoacetone	MAA
Nicotinamide Adenine Dinucleotide	NADH

Full Name	Abbreviation
Nicotinamide adenine dinucleotide phosphate	NADPH
<i>ortho</i> -Chlorophenol	o-CP
Hydroxylated polychlorinated biphenyl	OH-PCB
Polychlorinated biphenyl	PCB
Perchloroethylene	PCE
Pentachlorophenol	PCP
Dichlorohydroquinone dioxygenase	PcpA
Pentachlorophenol-4-Monooxygenase	PcpB
Tetrachlorohydroquinone reductive dehalonase	PcpC
Tetrachlorobenzoquinone reductase	PcpD
Maleylacetate reductase	PcpE
Reductive dehalogenase	RD
Trichloroethylene	TCE
Tetrachlorohydroquinone	TCHQ
tetrachlorophenol	TCP
Trichlorohydroquinone	TriCHQ
trichlorophenol	TriCP
Vinyl chloride	VC

CHAPTER ONE

Bacterial Reductive Dehalogenases^a

1.1 General Introduction

Toxic halogenated organic compounds originate from both natural (Gribble 1994, Gribble 1996) and industrial sources (Fetzner 1998, Mohn and Tiedje 1992a). In spite of the toxicity of these compounds, some bacteria have evolved mechanisms to metabolize them. A key group of bacterial enzymes that initiate the degradation of halo-organic compounds is the dehalogenases. Many types of dehalogenases have been described. They include mono-oxygenases, dioxygenases, dehydrohalogenases, reductive dehalogenases, hydrolytic dehalogenases, thiolytic dehalogenases, and methyl transferases (Fetzner 1998). This chapter concentrates on the microbial reductive dehalogenases. These enzymes are incorporated in three different microbial pathways that are involved in the degradation of halogenated compounds: co-metabolic, energy conservation, and carbon metabolism.

Co-metabolic or fortuitous dehalogenation processes do not directly benefit microorganisms because they are unable to derive any metabolic energy from these reactions. In fact, many co-metabolic reactions consume energy. The only benefit to the microorganism may arise indirectly from detoxification of the substrate(s) being co-metabolically dehalogenated. Co-metabolic dehalogenation reactions occur as a result of

^a A condensed version of this chapter has been accepted for publication. The full citation is: Habash, M.B., J.T. Trevors & H. Lee (2003) Bacterial reductive dehalogenases *In: Soil Biology: Bioremediation, Phytoremediation, and Natural Attenuation*. Eds. A. Singh & O.P. Ward. Springer-Verlag GmbH & Co. KG, Heidelberg, Germany (in press)

the broad substrate specificity, or “promiscuity”, of some microbial dehalogenases. Co-metabolic processes are found in many methanogenic, acetogenic, sulfate-reducing and iron-reducing microorganisms and involve mainly haloalkanes. These reactions may involve different cofactors such as corrinoid/Iron-Sulfur (FeS) or factor F₄₃₀, a nickel porphinoid co-factor found in methanogens (Holliger and Schraa 1994). Co-metabolic reactions have been reviewed elsewhere (Beaudette et al. 2002, El Fantroussi et al. 1998, Fetzner 1998, Holliger and Schraa 1994) and the reader is referred to those reviews for additional information.

This chapter reviews the biochemical, genetic and functional characteristics of the reductive dehalogenases involved in energy conservation and carbon metabolism pathways and, where possible, their regulation and proposed mechanisms of action. It will begin with a general description of the reductive dehalogenation mechanisms utilized by anaerobic and aerobic microorganisms. Next, the relevant toxic halogenated compounds and a review of the tetrachlorohydroquinone reductive dehalogenase that forms the foundation of the research undertaken in this thesis is presented. Subsequently, a general review of bacterial reductive dehalogenases involved in degrading halogenated compounds specifically for the benefit of a microorganism by energy conservation and carbon metabolism pathways are presented. With respect to energy conservation, some of the best-studied reductive dehalogenases found in anaerobic microorganisms including the 3-chlorobenzoate, tetra- and tri-chloroethene and chlorophenol reductive dehalogenases are explored (see Table 1.1). This is where most research relating to the reductive dehalogenases in anaerobic microorganisms resides and, together with our research involving an aerobic microorganism, provides a more complete understanding of

the family of reductive dehalogenases. With respect to carbon metabolism, many examples have been discovered (see Table 1.2), however, the focus herein is on two of the best-studied bacterial reductive dehalogenases that are also members of the glutathione transferase (GST) superfamily of enzymes, the dichloromethane dehalogenase and tetrachlorohydroquinone reductive dehalogenase. The chapter concludes with the research objectives and hypotheses examined in this thesis.

Table 1.1 Purified reductive dehalogenases from anaerobic microorganisms involved in dehalorespiration

Microorganism	Enzyme	MW and Number of Subunits	Cellular Localization	pH and Temperature optima	Substrates	Chromophore	Reference(s)
<i>Desulfomonile tiedjei</i> DCB-1	3-CBRD	Heterodimer 64 and 37 kDa	Membrane-bound	38°C 7.2-7.5	3-CB, PCE ^a , TCE ^a , PCP ^a and several other CPs ^a	Heme	(Mohn and Kennedy 1992b, Ni et al. 1995, Townsend and Suflita 1996)
<i>Desulfotobacterium chlororespirans</i>	Cl OHCB RD	50 kDa	Membrane-bound	6.8 59°C	o-CP, PCP, Cl OHCB, 2,3-, 2,6-DCP, 3,5 DCl OHB, OH PCB	Corrinoid and 2 FeS clusters	(Krasotkina et al. 2001)
<i>Desulfotobacterium dehalogenans</i>	o-CP RD	48 kDa Monomer	Membrane-bound	8.2 52°C	Cl OHPA, 2 CP, 2,3-, 2,4-, 2,6- DCP, PCP, 2B-4CP, OH-PCB	Corrinoid and 2 FeS clusters	(van de Pas et al. 1999, Wiegel et al. 1999)
<i>Desulfotobacterium</i> sp. strain PCE1	Cl OHPA RD	48 kDa Monomer	Membrane-associated ^c	nr	o-CP	Corrinoid and 2 FeS clusters	(van de Pas et al. 2001b)
<i>Desulfotobacterium</i> sp. strain PCE1	PCE RD	48 kDa Monomer	Membrane-associated ^c	nr	PCE, TCE	Corrinoid and 2 FeS clusters	(van de Pas et al. 2001b)
<i>Desulfotobacterium fragipieri</i> TCE1	PCE RD	59 kDa Monomer	Membrane-associated ^c	nr	PCE, TCE	Corrinoid and 2 FeS clusters	(van de Pas et al. 2001b)
<i>Desulfotobacterium</i> sp. strain PCE-S	PCE RD	65 kDa Monomer	Membrane-associated on cytoplasmic side	7.2 50°C	PCE, TCE	Corrinoid and 2 FeS clusters	(Miller et al. 1998)
<i>Desulfotobacterium</i> sp. strain Y51	PCE RD	58 kDa Monomer	periplasm	7.5, 37°C	PCE, TCE, hexa-, penta-, and tetra-CEAs	Corrinoid and 2 FeS clusters	(Suyama et al. 2002)
<i>Dehalospirillum multivorans</i>	PCE RD	57 kDa Monomer	cytoplasm	8 42°C	PCE, TCE, chlorinated propenes	Corrinoid and 2 FeS clusters	(Neumann et al. 1996, Neumann et al. 2002)
<i>Clostridium bifermentans</i>	PCE RD	35 and 35.7 kDa Dimer	Periplasm Membrane	7.5 35°C	DCE, TCE, cis-DCE, trans 1,2 ethylene, 1,1 DC ethylene	Corrinoid and 2 FeS clusters	(Okeke et al. 2001)
<i>Desulfotobacterium hafnense</i>	PCE RD	46.5 kDa	Membrane associated	nr	1,2 C propane PCE	Corrinoid and 2 FeS clusters ^b	(Christiansen et al. 1998)
<i>Dehalobacter restrictus</i>	PCE RD	61 kDa	Membrane associated on cytoplasmic side	nr	PCE	Corrinoid and 2 FeS clusters	(Schumacher et al. 1997)
<i>Dehalococcoides ethenogenes</i> 195	PCE RD	51 kDa Monomer	Membrane-bound	nr	PCE	Corrinoid and 2 FeS clusters	(Magnuson et al. 1998)
<i>Dehalococcoides ethenogenes</i> 195	TCE RD	61 kDa Monomer	Membrane-bound	nr	TCE, cis DCE, trans DCE, VC	Corrinoid and 2 FeS clusters	(Magnuson et al. 1998)

^a Co-metabolic dehalogenation induced by 3-chlorobenzoate; ^b likely 2 FeS clusters (Smidt et al. 2000b), however, quantification of Fe and S content of *D. hafnense* indicates potential for 3 FeS clusters (Christiansen et al. 1998); ^c nature of association with membrane was not determined (van de Pas et al. 2001b). Abbreviations: 2B-4CP: 2-bromo-4-chlorophenol; 3-CB: 3-chlorobenzoate; Cl OHPA: chlorohydroxyphenylacetate; CP: chlorophenol; DCE: dichloroethene; DClOHB: dichlorohydroxybenzoate; DCP: dichlorophenol; nr: not reported; OH-PCB: hydroxylated polychlorinated biphenyl; PCE: tetrachloroethene; PCP: pentachlorophenol; RD: reductive dehalogenase; TCE: trichloroethene; VC: vinyl chloride.

Table 1.2 Selected reductive dehalogenases from aerobic microorganisms involved in carbon metabolism of halogenated compounds

Organism	Enzyme	MW and Number of Subunits	Cellular Localization	pH and Temperature optima	Co-substrates	Substrates	Type of Reaction	Reference(s)
<i>Sphingomonas paucimobilis</i> UT26	2,5 DCHQ RD	40 kDa	Cytoplasm	nr	GSH	2,5 DCHQ, CHQ	Substitution via glutathione transfer	(Miyauchi et al. 1998)
<i>Sphingobium chlorophenolicum</i>	TCHQ RD	58 kDa Dimer (2x28 kDa monomers)	Cytoplasm	7-8 40°C	GSH	TCHQ, TriCHQ	Substitution via glutathione transfer	(Anandarajah et al. 2000, Kiefer and Copley 2002a, Xun 1992a, Xun et al. 1992b)
<i>Sphingomonas</i> sp. UG30	TCHQ RD	58 kDa Dimer (2x28 kDa monomers)	Cytoplasm	8.7 50°C	GSH	TCHQ, TriCHQ	Substitution via glutathione transfer	(Habash et al. 2002)
<i>Methylobacterium dichloromethanicum</i> DM4	DCM RD	37.4 kDa Monomer	Cytoplasm	8.0-8.5	GSH	DCM	Substitution via glutathione transfer	(La Roche and Leisinger 1990)
<i>Methylophilus</i> sp. DM11	DCM RD	31.2 kDa Monomer	Cytoplasm	8.0-10.0	GSH	DCM	Substitution via glutathione transfer	(Bader and Leisinger 1994, Vuilleumier et al. 2001)
<i>Hyphomicrobium</i> sp. DM2	DCM RD	nr	Cytoplasm	8.0-8.5	GSH	DCM	Substitution via glutathione transfer	(Kohler-Staub and Leisinger 1985, Vuilleumier et al. 2001)
<i>Methylohabdus multivorans</i> DM13	DCM RD	nr	Cytoplasm	8.0-9.0	GSH	DCM	Substitution via glutathione transfer	(Vuilleumier et al. 2001)
<i>Hyphomicrobium</i> sp. GJ21	DCM RD	nr	Cytoplasm	7.5-9.0	GSH	DCM	Substitution via glutathione transfer	(Vuilleumier et al. 2001)
<i>Corynebacterium sepedonicum</i> KZ-4	2,4-DCB-CoA RD	nr	Cytoplasm	nr	NADPH	2,4 DCB-CoA	Hydrolytic chloride elimination ^a	(Romanov and Hausinger 1996)
<i>Coryneform bacterium</i> strain NTB-1	2,4 DCB-CoA RD	nr	Cytoplasm	nr	NADPH	2,4 DCB-CoA	Hydrolytic chloride elimination ^a	(Romanov and Hausinger 1996)
<i>Rhodospseudomonas palustris</i>	3-CB-CoA RD	nr	Cytoplasm	nr	nr	3-CB-CoA	nr	(Egland et al. 2001)

^a speculated reaction, precise reaction is unclear (Romanov and Hausinger 1996)

Abbreviations: 3-CB-CoA: 3-chlorobenzoyl CoA; 2,4 DCB CoA: 2,4 dichlorobenzoyl CoA; DCHQ: dichlorohydroquinone; DCM: dichloromethane; GSH: glutathione; nr: not reported; RD: reductive dehalogenase; TCHQ: tetrachlorohydroquinone; TriCHQ: trichlorohydroquinone.

1.2 General Reductive Dehalogenation Mechanisms

Reductive dehalogenation reactions catalyzed by bacterial enzymes, known as reductive dehalogenases replace a halogen substituent, typically a chlorine, bromine or iodine atom, with a hydrogen atom. The reaction requires a reduced electron donor, a role fulfilled by a reduced organic substrate or H_2 . If the electron donor has sufficient reducing power, the removal of a chlorine atom can occur as a one-step reaction in which the electron donor provides both the two electrons and single proton necessary for the reaction (Mohn and Tiedje 1992a). Alternatively, if the electron donor does not have sufficient reducing power, the halogen atom is removed in a two-step reaction whereby the electron donor provides two electrons and the surrounding solvent, such as water, provides the proton (Mohn and Tiedje 1992a). The two-step reaction has been shown during reductive dechlorination of PCB (2,3,4,5,6 pentachlorobiphenyl) by vitamin B_{12} (Assaf-Anid et al. 1992) and in solvent kinetic studies with the reductive dehalogenase from *Desulfitobacterium chlororespirans* (Krasotkina et al. 2001). Overall, dehalogenation reactions can occur via the removal of either one or two halogen atoms (Figure 1.1). In the former, a single halogen atom can be replaced by hydrogen (Mohn and Tiedje 1992a), but in the latter, the removal of two adjacent halogen atoms results in the formation of a double bond between the carbons initially bonding the halogen groups (Mohn and Tiedje 1992a).

Figure 1.1 General reductive dehalogenation mechanisms. (I) Substitution of a chlorine atom by a hydrogen atom during the reductive chlorination of 3-chlorobenzoate to benzoate; and (II) Removal of two chlorine atoms on adjacent carbons, resulting in the formation of a double bond in the reductive dechlorination of 2-chloroethane to ethene.

1.3 Toxic Halogenated Compounds

Many industrially produced polyhalogenated organic compounds such as pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), perchloroethylene (PCE), trichloroethylene (TCE) and dichloromethane (DCM) are major environmental and human health problems because of their toxicity and persistence in the environment. Of

particular interest to this thesis are the compounds degraded by the pentachlorophenol degradative pathway of various aerobic organisms, namely pentachlorophenol and the first metabolic product tetrachlorohydroquinone.

1.3.1 General information on pentachlorophenol (PCP)

Pentachlorophenol (PCP, Figure 1.4) is a highly chlorinated xenobiotic compound that is used primarily as a fungicide for wood preservation (Cline et al. 1989, McAllister et al. 1996). Within the last 25 years, the use of PCP has been restricted or banned in many countries because of its persistence in soil and water. U.S. and European Environmental Protection Agencies have listed pentachlorophenol as a priority pollutant. In Canada, the use of PCP is regulated by the Pest Control Products Act (Waite et al. 1998). As a suspected human carcinogen, PCP poses a serious risk to human and environmental health.

Figure 1.2 Molecular structure of pentachlorophenol.

With regard to human health several aspects of PCP are important to note. Apart from direct ingestion through contaminated food or water, the entry of PCP into human beings can occur, presumably, via percutaneous adsorption, as shown into Rhesus monkeys (Wester et al. 1993). As well, inhalation via atmospheric spread of PCP has been implicated in the entry of PCP into human beings despite the lack of a direct source of PCP, such as a nearby industrial area that utilizes PCP (Waite et al. 1998). In indoor environments this is even more crucial as the persistence of PCP in treated wood results in PCP-laden dust that can be inhaled (Meissner and Schweinsberg 1996, Wilson et al. 2003). In two studies that examined the effect of a wood preserving plant on a nearby neighbourhood, in California, it was found that emissions from the plant resulted in elevated atmospheric levels of several wood processing waste chemicals, including polychlorinated dioxins (with PCP as their source), furans, and polycyclic aromatic hydrocarbons (Dahlgren et al. 2003a). In addition, exposed residents adjacent to the plant were found to have statistically significantly higher than average incidents of cancer, respiratory, skin, and neurological health problems (Dahlgren et al. 2003b).

The illnesses caused by long-term exposure to PCP can result in specific cellular and molecular defects. PCP has been found to cause neurotoxic effects in combination with an organophosphate (mirapox), as measured by inhibition of acetylcholinesterase production in SY5Y human neuroblastoma cells (Tang et al. 2003); however, the principal mechanism by which PCP causes geno- or cyto-toxicities within the human body is via its metabolite TCHQ. The cellular toxicity of TCHQ is caused by its ability to form DNA adducts (Lin et al. 2001a, Lin et al. 2001b); encourage the formation of reactive oxygen species (Naito et al. 1994); deplete stores of protective cellular

glutathione through conjugation with TCHQ (Wang et al. 1997); and increase cellular levels of p53, a protein involved in programmed cell death (Wang et al. 1997).

In aquatic environments, PCP becomes adsorbed to sediments and suspended particles (Abramovitch and Capracotta 2003), where it has been detected at low levels (0.01–16 $\mu\text{g/l}$) in rivers, streams, surface water systems and seawater (Howard and Meylan 1997). In soils, PCP adsorption has been found to increase as the organic matter content of the soil increases (van Gestel and Ma 1988). The effect of PCP on these environments results in a change in diversity likely, by the ability of PCP to uncouple oxidative phosphorylation, thereby inhibiting the growth of many organisms (Trevors 1982). For example, the presence of PCP in a reactor containing soil slurry resulted in a decrease in microbial diversity selecting for a smaller population of bacteria capable of PCP-degradation or tolerance (Beaulieu et al. 2000). Furthermore, PCP has been shown to affect the community structure in humus-containing soil resulting in an adverse effect on decomposition and nutrient mobilization (Salminen et al. 1995).

An interesting aspect of PCP is the number of microorganisms that have evolved mechanisms to tolerate and/or degrade PCP (Table 1.3). This has occurred despite the toxicity of PCP and the fact that it has been present in the environment for only about 66 years (Cline et al. 1989) with no known natural analogs. Of particular interest are those microorganisms that have evolved the reductive dehalogenase PcpC.

Table 1.3 Selected microbial isolates known to degrade pentachlorophenol

Anaerobic bacteria	Metabolic Products	References
<i>Desulfomonile tiedjei</i> DCB-1	PCP to 2,4,6 TriCP	(Mohn and Kennedy 1992b)
<i>Desulfitobacterium frappieri</i> strain PCP-1	PCP to 3-CP	(Beaudet et al. 1998, Bouchard et al. 1996)
<i>Desulfitobacterium dehalogenans</i> JW/IU-DC1	PCP to TCP	(Utkin et al. 1995, van de Pas et al. 1999)
Aerobic bacteria	Metabolic Products	References
<i>Saccharomonospora viridis</i>	PCP to TCHQ	(Webb et al. 2001)
<i>Mycobacterium chlorophenolicus</i> PCP-1 (formerly <i>Rhodococcus chlorophenolicus</i> PCP-1)	PCP to CO ₂ and H ₂ O	(Apajalahti and Salkinoja-Salonen 1987, Brandt et al. 1999, Briglia et al. 1994, Uotila et al. 1991, Uotila et al. 1995)
<i>Sphingomonas</i> sp. UG30 (formerly <i>Pseudomonas</i> sp. UG30)	PCP to CO ₂ and H ₂ O	(Leung et al. 1997)
<i>Sphingomonas</i> sp. UG25 (formerly <i>Pseudomonas</i> sp. UG25)	PCP to CO ₂ and H ₂ O	(Leung et al. 1997)
<i>Sphingobium chlorophenolicum</i> sp. strain RA2 (formerly <i>Pseudomonas</i> and <i>Sphingomonas</i> sp. strain RA2)	PCP to CO ₂ and H ₂ O	(Colores et al. 1995, Colores and Schmidt 1999, Ederer et al. 1997, Radehaus and Schmidt 1992, Schmidt et al. 1999)
<i>Sphingomonas</i> sp. strain ATCC 33790 (formerly <i>Arthrobacter</i> sp. strain ATCC 33790)	PCP to CO ₂ and H ₂ O	(Ederer et al. 1997, Schenk et al. 1989, Schenk et al. 1990, Stanlake and Finn 1982)
<i>Sphingomonas</i> sp. strain SR3 (formerly <i>Pseudomonas</i> sp. strain SR3)	PCP to CO ₂ and H ₂ O	(Ederer et al. 1997, Resnick and Chapman 1994)
<i>Sphingobium chlorophenolicum</i> ATCC 39723 (formerly <i>Flavobacterium</i> , and <i>Sphingomonas chlorophenolica</i> ATCC 39723)	PCP to CO ₂ and H ₂ O	(Ederer et al. 1997, Saber and Crawford 1985, Takeuchi et al. 2001, Topp et al. 1988, Topp and Hanson 1990)
<i>Streptomyces rochei</i> 303	PCP to CO ₂ and H ₂ O	(Golovleva et al. 1991, Zaborina et al. 1997)
<i>Rhodococcus</i> sp. strain CG-1	PCP to CO ₂ and H ₂ O	(Haggbloom et al. 1988)
<i>Mycobacterium</i> sp. strain CG-2	PCP to CO ₂ and H ₂ O	(Haggbloom et al. 1988)
<i>Rhodococcus</i> sp. strain CP-2	PCP to CO ₂ and H ₂ O	(Haggbloom et al. 1988)
<i>Pseudomonas</i> sp. Bu34	PCP to CO ₂ and H ₂ O	(Lee et al. 1998)
<i>Pseudomonas veronii</i> PH-05	PCP to tetrachlorocatechol	(Nam et al. 2003)
<i>Trichoderma harzianum</i>	PCP to pentachloroanisole	(Rigot and Matsumura 2002)
<i>Pseudomonas</i> sp. strain IST103	PCP to CHQ	(Thakur et al. 2001)
<i>Novosphingobium</i> sp. strain MT1	PCP to CO ₂ and H ₂ O	(Tirola et al. 2002)
<i>Pseudomonas cepacia</i> AC1100	PCP to TCHQ	(Tomasi et al. 1995)

Abbreviations: PCP – pentachlorophenol; TCHQ – tetrachlorohydroquinone; TCP – tetrachlorophenol; CP - chlorophenol; TriCP – trichlorophenol;

1.4 Tetrachlorohydroquinone reductive dehalogenase (PcpC)

One of the reductive dehalogenases and the focus of this thesis, tetrachlorohydroquinone reductive dehalogenase (PcpC), is involved in the bacterial degradation of PCP. This enzyme provides an excellent example of a reductive dehalogenase that works in concert with several other enzymes in a dehalogenation pathway involved in carbon metabolism.

1.4.1 General characteristics of PcpC

Present in several PCP-degrading aerobic bacteria (Crawford and Ederer 1999, Haggblom et al. 1988, Haggblom et al. 1989, Leung et al. 1997, Saber and Crawford 1985, Uotila et al. 1991, Xun et al. 1992b), PcpC has been best characterized from *Sphingobium chlorophenolicum* ATCC 39723. The enzyme is a 58 kDa homodimer able to catalyze two very different glutathione-dependent reactions (Figure 1.3): an isomerization (Anandarajah et al. 2000) and a reductive dechlorination (Anandarajah et al. 2000, Habash et al. 2002, McCarthy et al. 1996, Xun et al. 1992b). In vivo, the isomerization reaction converts maleylacetoacetone (MAA) to fumarylacetoacetone, an intermediate step in phenylalanine and tyrosine catabolism (Anandarajah et al. 2000). Because of the instability of MAA, however, in vitro experiments examining PcpC isomerase activity utilized maleylacetone (Anandarajah et al. 2000), which is an acceptable MAA alternative (Seltzer 1973). The reductive dechlorination occurs in two successive steps. First, tetrachlorohydroquinone (TCHQ) is converted to trichlorohydroquinone (TriCHQ) by the replacement of a chlorine atom with hydrogen.

Second, TriCHQ is converted to dichlorohydroquinone (DCHQ) with the replacement of a second chlorine atom with hydrogen (McCarthy et al. 1996, Xun et al. 1992b). These two reactions form part of the PCP degradation pathway in these bacteria. Aromatic substrates attacked by PcpC must have two hydroxyl groups *para* to each other. Pentachlorophenol and tetrachlorophenol are competitive inhibitors of this reaction; however, the lack of *para*-substituted hydroxyl groups in these compounds render them unable to be turned over by PcpC (Kiefer and Copley 2002a).

Figure 1.3 The two glutathione-dependent reactions catalyzed by PcpC. (I) The isomerization of maleyl acetoacetate to fumaryl acetoacetate; and (II) the two reductive dechlorination steps forming dichlorohydroquinone from tetrachlorohydroquinone via trichlorohydroquinone (Anandarajah et al. 2000).

1.4.2 Reaction mechanism

A reaction mechanism for the *S. chlorophenolicum* PcpC (Figure 1.4) has been proposed by McCarthy et al. (1996), Kiefer et al. (2002), and Kiefer and Copley (2002). The initial steps catalyzed by PcpC to conjugate reduced glutathione to TCHQ do not follow the typical nucleophilic substitution reaction seen in most GSTs (Kiefer et al. 2002b). Rather, it has been shown that this reaction involves the ketonization of deprotonated TCHQ to form 2,3,5,6-tetrachloro-4-hydroxycyclohexa-2,4-dienone, followed by a 1,4-elimination reaction to form trichlorobenzoquinone (Kiefer and Copley 2002a). Trichlorobenzoquinone then reacts with reduced glutathione to form a glutathione conjugate (Kiefer and Copley 2002a).

In subsequent steps, glutathione is removed by formation of a mixed disulfide between glutathione and an N-terminal Cys from PcpC (McCarthy et al. 1996). The enzyme utilizes Cys13 in the glutathione binding site to act as a nucleophile to remove the conjugated glutathione during catalysis (McCarthy et al. 1996, Willett and Copley 1996). A second reduced glutathione is used to extract the enzyme-bound glutathione to form oxidized glutathione. This is only one of several known examples of GSTs that utilize two molecules of reduced glutathione (GSH) for the removal of a halogen group with the production of oxidized glutathione (Kayser et al. 2002). The other examples, also bacterial enzymes, are the dichlorohydroquinone reductive dehalogenase (LinD) from *Sphingomonas paucimobilis* strains UT26 (Miyauchi et al. 1998) and B90 (Kumari et al. 2002), and LigF from *Pseudomonas paucimobilis* SYK-6 (Masai et al. 1993).

2,3,5,6 tetrachloro-4-hydroxycyclohexa-2,4-dienone

HCl

Trichlorobenzoquinone

-S-Enz
Cys13

GSSEnz
Cys13

GSSG

Trichlorohydroquinone

2,3,5 trichloro-S-glutathionyl-4-hydroxycyclohexa-2,4-dienone

Figure 1.4 Proposed mechanism for the removal of the first chlorine atom from tetrachlorohydroquinone (TCHQ) by PcpC as described by Kiefer *et al.* (2002) and McCarthy *et al.* (1996).

1.4.3 Molecular characterization

PcpC is a member of the pathway that initiates pentachlorophenol degradation in *S. chlorophenolicum* (Figure 1.5). All genes in this initial pathway have been cloned and sequenced and the organization and regulation of the genes has been examined. The catabolic gene clusters were localized to two fragments. The first 24-kb fragment contains approximately twenty ORFs, four of which were involved in PCP degradation (Cai and Xun 2002). Two of the four genes have been identified. They are *pcpC*, encoding the GST responsible for reductive dechlorination of TCHQ and TriCHQ, and *pcpA*, encoding the ring cleaving 2,6-dichlorohydroquinone 1,2-dioxygenase. Two recently identified genes are *pcpE*, encoding maleylacetate reductase, and *pcpM*, a proposed regulatory gene whose role in PCP degradation remains unknown (Cai and Xun 2002). The second 8-kb fragment contains the previously identified *pcpB* encoding PCP-4-monoxygenase, *pcpD* encoding TCHQ reductase, and *pcpR*, a LysR type regulatory gene required for PCP degradation (Cai and Xun 2002).

The organization of the genes is unusual in that the five catabolic genes, *pcpA*, *pcpB*, *pcpC*, *pcpD* and *pcpE*, are localized in four discrete locations in the genome (Cai and Xun 2002). Another interesting feature is the expression of *pcpA*, *pcpB*, *pcpD* and *pcpE* is inducible by PCP while the expression of *pcpC* is constitutive (Cai and Xun 2002). Two reasons have been proposed to explain the constitutive expression of this gene. First, PcpC may have other non-essential functions in the cell that have not been identified (Cai and Xun 2002). Another explanation for the constitutive nature of PcpC may be found in its proposed evolution from a maleyl acetoacetate isomerase (Copley 2000). Maleyl acetoacetate isomerase is a central metabolic pathway enzyme involved in

tyrosine metabolism and constitutive expression of central metabolic pathway genes is not unusual. Alternatively, maleyl acetoacetate isomerase expression may have been regulated by elements able to recognize the presence of tyrosine or one of its metabolites. It should, however, be recognized that during the evolution of PcpC, and the PCP degradative pathway, these maleyl acetoacetate isomerase regulatory elements would have failed to recognize PCP to initiate production of PcpC. The spontaneous mutation of the maleyl acetoacetate regulatory elements and the subsequent selection of constitutively expressed *pcpC* would have been a possible event (Cai and Xun 2002).

1.4.4 Structural characterization: Comparison to other GSTs

Structural data on the canonical GST classes has been recently reviewed (Sheehan et al. 2001). Despite a low level of sequence homology, GSTs from all classes have a basic scaffold. Each monomer of the enzyme consists of two domains. The N-terminal domain consists of the first 80 amino acids and takes the form of a highly conserved thioredoxin fold that is responsible for GSH binding (Sheehan et al. 2001). The second domain, made up of α -helices, is primarily responsible for binding the second substrate (Sheehan et al. 2001). The second domain can be more variable because of the broad substrate range of the different classes of GST. The two domains are joined by a short amino acid linker sequence.

Figure 1.5 The pentachlorophenol degradative pathway proposed for *Sphingomonas* sp. UG30, as extrapolated from the *S. chlorophenolicum* ATCC 39723 pathway. The pathway was adapted from http://umbbd.ahc.umn.edu/pcp/pcp_image_map.html. The PcpD reaction is described by (Dai et al. 2003).

PcpC is presently classified as a member of the Zeta class of the GST superfamily based on sequence analyses and substrate range (Anandarajah et al. 2000). To date, PcpC has not yet been crystallized for three-dimensional structural determination. Because the GSTs have a common structure, an examination of other crystallized members of the same class may provide some insight into the structural features of PcpC important for activity. One example of a crystallized Zeta class GST is the human maleyl acetoacetate isomerase, GSTZ1 (Polekhina et al. 2001). One of the amino acids proposed to be important for activity is an N-terminal Ser which stabilizes GSH in the glutathione-binding domain (Board et al. 1997, Polekhina et al. 2001). Although this residue normally points away from the active site, it has been suggested that on GSH binding, a conformational change may occur to allow orientation in the proper direction, as has been shown in the Pi GST class (Oakley et al. 1997).

The biochemical (maleylacetone isomerization and dichloroacetic acid metabolism) and primary sequence similarities of PcpC to GSTZ1 indicate that PcpC may have evolved from an ancient pathway of tyrosine catabolism (Anandarajah et al. 2000, Polekhina et al. 2001). Relatively recent mutations would have allowed PcpC to accept the substrates TCHQ and TriCHQ, which are derived from the degradation of PCP, despite its recent introduction in the environment. As well, *S. chlorophenolicum* possesses at least one other *bona fide* maleyl acetoacetate isomerase. Thus, it was suggested that a gene duplication followed by mutagenesis of the duplicated gene might have resulted in the initial development of PcpC to be recruited for use in the PCP degradation pathway. The exact evolutionary origin of PcpC, however, is not known.

The *Sphingobium* PcpC has been suggested to be an immature enzyme as it

suffers from severe substrate inhibition (Anandarajah et al. 2000, Copley 1998, Copley 2000). *In vitro*, this has hampered efforts to accurately determine the kinetic parameters of the wildtype enzyme (Anandarajah et al. 2000, Kiefer and Copley 2002a, Kiefer et al. 2002b, McCarthy et al. 1996), though the substrate concentration where this inhibition occurs has not been reported. *In vivo*, the impact of the substrate inhibition is difficult to evaluate. It has been estimated that *in vivo* concentrations of TCHQ in *Sphingobium chlorophenolicum* cells exposed to 0.94 mM PCP for 50 minutes reached 9 μ M, although cytoplasmic concentrations were lower at 2 μ M (McCarthy et al. 1997). If substrate inhibition does not occur at such low concentrations, there would likely be no selective pressure for cells to evolve an enzyme with less substrate inhibition.

1.5 Reductive Dehalogenases Involved in Energy Conservation

Some bacteria can couple the degradation of halogenated organic compounds to the production of cellular energy. This process, termed dehalorespiration, has only been found in anaerobic microorganisms and includes the well-studied dehalogenation reactions of tetrachloroethene (PCE), trichloroethene (TCE), chlorophenols (CP), and 3-chlorobenzoate (3-CB). The basis for energy conservation in bacteria is the development of an electrochemical gradient on either side of a membrane (a chemiosmotic gradient), produced by the translocation of protons and/or their utilization on one side of a membrane coupled with their production on the other side. This is associated with the passage of electrons through an electron transport chain present within the bacterial inner membrane. This is reminiscent of ATP production via the electron transport system

present within the inner membrane of mitochondria in eukaryotic cells.

In anaerobic bacteria, the removal of halogen atoms by reductive dehalogenases drives the dehalorespiration process by helping to form a proton gradient on either side of the bacterial inner membrane (Figure 1.2). Membrane-bound hydrogenases and/or formate hydrogenases produce protons (H^+) on the periplasmic side of the inner membrane, while on the cytoplasmic side, H^+ is utilized by a soluble or membrane-associated reductive dehalogenase. The presence of the reductive dehalogenase at the end of the electron transport chain allows its halogenated substrate to serve as a terminal electron acceptor. The incorporation of a proton and the electrons results in the release of the halogen atom. Thus, the dehalogenation pathway performs two important tasks. The first is the formation of a proton motive force (PMF), resulting in H^+ movement from the periplasmic to cytoplasmic sides of the membrane through an ATP-generating ATPase. The second is the dehalogenation of toxic halogenated compounds resulting in their complete or partial biodegradation. This pathway has been termed the chemiosmotic model of dehalorespiration and was first proposed by Tiedji and Mohn (1990) based on studies with *Desulfomonile tiedjei*, which possesses the 3-CB reductive dehalogenase.

1.5.1 3-Chlorobenzoate Dehalogenase

Desulfomonile tiedjei is a non-motile bacterium, isolated from an enrichment culture, that utilizes 3-CB as a terminal electron acceptor (Mohn and Tiedje 1990). Although not all of the components of the complete electron transport pathway have been identified, studies have identified several components that include a hydrogenase, formate dehydrogenase, a novel cytochrome c, a membrane-bound quinone of unknown

type and the terminal 3-CB reductive dehalogenase (Louie et al. 1997, Louie and Mohn 1999). Evidence has shown that a scalar mechanism, i.e. where protons are not translocated, establishes a PMF that results in ATP production. It has also been suggested that a translocation process takes place; however, this has not yet been verified (Louie and Mohn 1999).

An important component of this pathway is the inducible terminal 3-CB reductive dehalogenase, which was the first reductive dehalogenase to be purified from an anaerobic bacterium (Ni et al. 1995). This enzyme comprises two subunits (64 and 37 kDa) and is believed to be an integral membrane protein (Ni et al. 1995). Unlike other reductive dehalogenases, 3-CB dehalogenase possesses a heme group that acts as a redox center and is not adversely affected by oxygen (Ni et al. 1995). In addition to 3-CB, pure cultures of *D. tiedjei* appear to co-metabolically dehalogenate tetrachloroethene (PCE) (Townsend and Suflita 1996) and pentachlorophenol (PCP) (Mohn and Kennedy 1992b). The ability to dehalogenate PCE and PCP was induced in the presence of 3-CB, while neither TCE nor PCP alone induced dehalogenation of PCE and/or PCP by *D. tiedjei* (Mohn and Kennedy 1992b, Townsend and Suflita 1996). Furthermore, in cell suspensions of *D. tiedjei* in deuteriated water (D_2O) it has been shown that reductive dehalogenation occurs at the location where the halogen atom is removed. This indicates that a partial ring reduction, which would allow incorporation of a proton at a location other than the site of halogen removal, does not happen (Griffith et al. 1992). To date, no structural or mechanistic data have been presented for the purified enzyme.

Figure 1.6 Proposed dehalorespiratory pathways in anaerobic bacteria that are capable of growth on halogenated alkyl and aryl compounds (RCl). An electron transport system (ETS) with the reductive dehalogenase (RD) as the terminal enzyme utilizes the halogenated compounds as electron acceptors. The RD can be associated with the inner cell membrane as (I) an integral membrane protein, and (II) through attachment by a membrane anchor protein (MA). Other components include the H⁺ producing hydrogenase (H) and/or formate dehydrogenase (FD) present on the periplasmic side of the inner membrane. An ATPase translocates H⁺ from the periplasmic to the cytoplasmic side of the inner membrane. This reaction is coupled to the production of ATP.

Other anaerobic bacteria have been discovered that are capable of dehalorespiration. Some of these include various *Desulfitobacterium* sp. (Christiansen and Ahring 1996, Gerritse et al. 1999, Holliger et al. 1998, Miller et al. 1997, Sanford et al. 1996, Suyama et al. 2002, Utkin et al. 1995, van de Pas et al. 2001b), *Dehalobacter restrictus* (Holliger et al. 1998), *Clostridium bifermentans* DPH-1 (Okeke et al. 2001), *Dehalospirillum multivorans* (Neumann et al. 1994), and *Dehalococcoides ethenogenes* 195 (Maymo-Gatell et al. 1999). The common feature amongst these various anaerobic microorganisms is the presence of reductive dehalogenases that are the terminal enzymes in an electron transport chain involved in dehalorespiration. The inducible reductive dehalogenases from these anaerobic organisms remove halogen atoms from four main substrates: PCE, TCE, 3-chloro-4-hydroxybenzoate, and 3-chloro-4-hydroxyphenylacetate.

The reductive dehalogenases involved in dehalorespiration share many common characteristics. These enzymes have molecular weights ranging from 48 to 65 kDa, with optimal pH and temperature ranging from 7.2 to 8.2 and 37°C to 52°C, respectively. They are all inhibited by oxygen and require an electron donor, such as methyl viologen, for activity. The physiological electron donors are not known. These enzymes possess identical redox centers, comprised of a cobalamin-containing corrinoid factor and iron-sulfur clusters. In addition, these enzymes are typically membrane-associated either as integral membrane proteins or peripherally through a separate anchoring protein that contains two or three membrane-spanning regions. The gene for the protein anchor, known as PceB or CprB, is found either upstream or downstream of the gene coding for the reductive dehalogenase and is co-transcribed with the dehalogenase.

Sequence analyses have revealed important conserved regions in all reductive dehalogenase-encoding genes. Two areas coding for the iron-sulfur clusters of the reductive dehalogenases are highly conserved. Typically, one iron-sulfur cluster is in the form of Fe_4S_4 (represented by the consensus sequence CXXCXXCXXXCP) and the other is in the form of Fe_3S_4 (represented by the consensus sequence GXXCXXCXXXCP). Many of the sequences contain the twin Arg signal sequence (RRXFXK), which plays a role in protein maturation and translocation of mainly periplasmic proteins that bind different redox cofactors (Berks 1996, Berks et al. 2000). Sequence analysis also indicated that the reductive dehalogenases from this group of microorganisms do not possess the consensus sequence for a corrinoid-binding motif (DXHXXG). This perhaps reflects the fact that the corrinoid binds non-covalently to the enzyme (Neumann et al. 1998).

1.5.2 3-Chloro-4-hydroxyphenylacetate Reductive Dehalogenase

1.5.2.1 General enzyme characterization

Separate reductive dehalogenases from *Desulfitobacterium dehalogenans*, *Desulfitobacterium hafniense*, and *Desulfitobacterium* sp. PCE1 have been identified that are induced by, and can degrade 3-chloro-4-hydroxyphenylacetate (Cl-OHPA). The enzyme from *Desulfitobacterium* sp. PCE1 will be described in the PCE/TCE reductive dehalogenase section (Section 1.3.4). One of the best-studied reductive dehalogenases in this group is from *Desulfitobacterium dehalogenans*, a strain first isolated from a freshwater sediment sample by enrichment on 2,4 dichlorophenol (2,4-DCP) (Kohring et

al. 1989a, Kohring et al. 1989b). The strain was capable of 2,4-DCP and Cl-OHPA dechlorination utilizing Cl-OHPA as an electron acceptor. In addition, *D. dehalogenans* can dehalogenate a variety of penta-, tetra-, tri-, and di-chlorophenols when grown with either Cl-OHPA or 2,4 DCP as inducers of reductive dehalogenation (Utkin et al. 1995). Experiments involving whole and broken cells with methyl viologen, an electron donor unable to penetrate the cell membrane, indicated the dehalogenating activity was present in the cytoplasm (van de Pas et al. 1999). The ability of *D. dehalogenans* to reductively dehalogenate *ortho*-chlorophenols is linked to a 48 kDa membrane-associated *ortho*-chlorophenol (o-CP) reductive dehalogenase (van de Pas et al. 1999). The substrate range of the o-CP reductive dehalogenase includes Cl-OHPA, PCP, several dichlorophenols (2,3-, 2,4-, and 2,6-DCP), and 2-chlorophenol (van de Pas et al. 1999). *D. dehalogenans* can reductively dechlorinate the para-hydroxylated PCB derivatives 4-hydroxylated PCB and 4,4'-dihydroxylated PCB (Wiegel et al. 1999); however, it is not known if o-CP reductive dehalogenase is specifically responsible for this activity. No measurable activity has been observed against tetrachloroethene or trichloroethene (van de Pas et al. 1999).

The energy-producing pathway in *D. dehalogenans* possesses many proteins with functions similar to the 3-CB dehalorespiratory pathway of *D. tiedjei*. The o-CP reductive dehalogenase is likely the terminal reductase involved in the energy-producing electron transport system that utilizes o-CP as an electron acceptor. Only a fraction of the theoretically possible ATP (El Fantroussi et al. 1998) is produced, however, indicating an inefficient energy-producing pathway (van de Pas et al. 2001a). Other components of the electron transport chain of *D. dehalogenans* have been examined using random Tn916

insertion to create various dehalorespiring mutants (Smidt et al. 1999). H₂-hydrogenase and formate dehydrogenase mutants were found to be incapable of dehalorespiration, likely because of the disruption of an electron transport system required for dehalorespiration by *D. dehalogenans* (Smidt et al. 1999).

A second Cl-OHPA reductive dehalogenase has been purified from *Desulfitobacterium hafniense*, a strain isolated from an enrichment culture obtained from a trichlorophenol-converting microbial consortium from municipal sludge (Christiansen and Ahring 1996). The purified enzyme was similar to the o-CP reductive dehalogenase from *D. dehalogenans* in several ways. The enzyme can reductively dehalogenate Cl-OHPA, but not PCE (Christiansen et al. 1998). The 47-kDa membrane-associated dehalogenase contains a corrinoid factor as well as iron sulfur clusters (Christiansen et al. 1998). One difference between the reductive dehalogenase from *D. hafniense* and all other reductive dehalogenases is the calculated values of iron and sulfur present in the enzyme. The *D. hafniense* reductive dehalogenase was determined to have twelve iron and twelve sulfur atoms per subunit (Christiansen et al. 1998). In contrast, all other reductive dehalogenases, with the exception of the 3-CB reductive dehalogenase from *D. tiedjei* that does not possess any FeS clusters, have seven to eight iron and eight sulfur atoms per subunit. The authors suggest this may indicate the presence of three Fe₄S₄ clusters in the *D. hafniense* reductive dehalogenase (Christiansen et al. 1998). Based on the amino acid sequence of the o-CP reductive dehalogenase, however, the *D. hafniense* enzyme contains the conserved FeS sequences found in other reductive dehalogenases, suggesting that it may contain only two FeS clusters (Smidt et al. 2000b). This apparent discrepancy remains to be investigated.

1.5.2.2 Molecular characterization

Currently, the *D. hafniense* genome is being sequenced. An examination of the primary sequences of the o-CP reductive dehalogenases (CprA) from *D. hafniense*, *D. dehalogenans* and *Desulfitobacterium* PCE1 show nearly identical sequences, sharing approximately 99% identity (Smidt et al. 2000b).

The entire o-CP gene cluster from *D. dehalogenans* has been characterized. The o-CP reductive dehalogenase, encoded by the *cprA* gene, is responsible for dehalogenating activity towards o-CP (van de Pas et al. 1999). An examination of the CprA primary sequence reveals features typical for this class of reductive dehalogenase, including two iron-sulfur clusters, one Fe₄-S₄ ferredoxin-like center and one Fe₃-S₄ truncated iron-sulfur binding cluster binding motif, and a cobalamin-containing center per monomer (van de Pas et al. 1999). Also, characteristic of periplasmic redox center containing proteins, a twin Arg-type signal sequence is present in CprA (van de Pas et al. 1999). Upstream of *cprA* is the *cprB* gene coding for the small hydrophobic anchor protein (van de Pas et al. 1999).

Several other genes, along with *cprA* and *cprB*, have been found that are likely to be involved in regulation of gene transcription and protein folding. *cprC* and *cprK* code for potential transcriptional regulators, while *cprD* and *cprE* code for proteins very similar to the GroEL chaperonins that aid in protein folding and prevention of protein aggregation (Smidt et al. 2000a). *cprT* codes for a protein with great similarity to a propyl peptidyl isomerase that catalyses the cis-trans isomerization of proline residues. This activity aids in protein folding (Smidt et al. 2000a). Comparison of the dehalorespiratory pathway of *D. dehalogenans* to others (Smidt et al. 2000a) indicates a similarity to the

proposed respiratory complexes found in denitrifying bacteria, which use an electron transport system for the reduction of nitrate to nitrogen gas (Moura and Moura 2001).

1.5.3 3-chloro-4-hydroxybenzoate Reductive Dehalogenase

1.5.3.1 General enzyme properties

An integral membrane dehalogenase induced by and capable of 3-chloro-4-hydroxybenzoate (Cl-OHCB) reductive dehalogenation has been found in *Desulfitobacterium chlororespirans*. This microorganism, isolated from an enrichment culture originating from compost soils, can dechlorinate 2,3-dichlorophenol (DCP) (Sanford et al. 1996) and is capable of growth on 2,3- and 2,6-DCP, 2,4,6-tribromophenol, Cl-OHCB, and Cl-OLPA, which serve as electron acceptors (Sanford et al. 1996). Dehalogenation is carried out by a 50-kDa membrane-bound protein with many characteristics similar to the other reductive dehalogenases involved in dehalorespiration (Krasotkina et al. 2001). This includes oxygen sensitivity, possible post-translational modification (since methionine was not the first amino acid), and the presence of the conserved sequence NYVPG, which is present in all published sequences of reductive dehalogenases isolated from *Desulfitobacterium* strains (Krasotkina et al. 2001). Currently, no role has been postulated for this conserved sequence. Other similarities involve the redox reaction centers present in the enzyme, including two FeS clusters and one corrinoid (Krasotkina et al. 2001). An important characteristic that distinguishes the *D. chlororespirans* reductive dehalogenase from others is its ability to dehalogenate hydroxylated PCBs. This is currently the only known enzyme with this capability (Krasotkina et al. 2001).

The proposed catalytic mechanism of this reductive dehalogenase involves donation of a proton by the solvent to aid in the removal of the chlorine atom from the substrate. Solvent kinetic experiments using $^2\text{H}_2\text{O}$ indicated that a partially rate-limiting step is an intramolecular proton transfer, with the proton originating from the surrounding solvent (Krasotkina et al. 2001). In addition, substrates with a hydroxyl group *ortho* to the chlorine are necessary for dehalogenase activity (Krasotkina et al. 2001). Competitive inhibitors to the enzyme also require this *ortho* hydroxyl configuration to be effective. The presence of another functional group *meta* to the chlorine was necessary for recognition of the substrate by the enzyme. It was shown that 2-chlorophenol was not a substrate or inhibitor, however, the addition of a meta substituted carboxyl or acetyl group, such as with Cl-OHCB and Cl-OHPA, resulted in dehalogenation of the substrate (Krasotkina et al. 2001). It is not certain why the *ortho* hydroxyl and meta substituent in the substrate are required for dehalogenase activity. It has been suggested that they may be involved in binding to the substrate pocket of the enzyme (Krasotkina et al. 2001). Further structural studies with these reductive dehalogenases may help answer this question.

1.5.4 Tetrachloroethene and Trichloroethene Reductive Dehalogenases

Many genera of anaerobic bacteria have been isolated with the ability to dehalogenate tetrachloroethene (PCE) and/or trichloroethene (TCE). These include several *Desulfitobacterium* species including strain PCE-S (Miller et al. 1997, Miller et al. 1998), PCE1 (van de Pas et al. 2001b), *D. frappieri* TCE1 (Gerritse et al. 1999), and strain Y51 (Suyama et al. 2002). All these microorganisms are capable of converting

PCE to TCE and then to cis-1,2-dichloroethene (DCE) except for strain PCE1 that only converts PCE to TCE.

The PCE reductive dehalogenases from *Desulfitobacterium* sp. PCE-S and *D. frappieri* TCE1 are very similar, with molecular weights of 65 and 59 kDa, respectively (Miller et al. 1998, van de Pas et al. 2001b). Furthermore, their N-terminal sequences are highly homologous (van de Pas et al. 2001b). The PCE-S dehalogenase has been shown to be bound to the membrane facing the cytoplasmic side (Miller et al. 1997). It is likely that the TCE1 dehalogenase is also membrane-bound. Another characteristic of these dehalogenases is their narrow chlorinated substrate range. Both enzymes only dehalogenate PCE and TCE, as shown in assays with cell extracts (Gerritse et al. 1999, Miller et al. 1997) and purified enzymes (Miller et al. 1998, van de Pas et al. 2001b). This is similar to other PCE/TCE reductive dehalogenases, such as those from strain PCE1 (van de Pas et al. 2001b) and *Dehalobacter restrictus* (Holliger et al. 1998, Schumacher et al. 1997).

An interesting member of the *Desulfitobacterium* genus is *Desulfitobacterium* sp. strain PCE1 which is able to dechlorinate both PCE and chlorophenols using two separately inducible enzymes (van de Pas et al. 2001b). As described earlier, *Desulfomonile tiedjei* is also capable of chlorophenol and PCE/TCE dehalogenation. The 3-CB reductive dehalogenase in *D. tiedjei* is involved in energy conservation during the utilization of 3-CB (Ni et al. 1995). Its ability to dehalogenate PCE/TCE has been shown to be because of co-metabolism, rather than dehalorespiration or dechlorination for carbon metabolism (Townsend and Suflita 1996).

Induction of strain PCE1 with either PCE or Cl-OHPA results in different protein

profiles and substrate ranges, suggesting different degradation pathways for each compound (van de Pas et al. 2001b). Cell extracts of strain PCE1 grown on PCE exhibit activity towards PCE and TCE, but not chlorophenols. Furthermore, growth on PCE induces the production of a 48 kDa enzyme that degrades PCE to TCE (van de Pas et al. 2001b). Degradation of TCE does occur, albeit at a rate corresponding to approximately 10% of the rate of PCE degradation. In comparison, the PCE reductive dehalogenase of strain TCE1 has nearly identical rates of degradation for PCE and TCE (van de Pas et al. 2001b). When grown on Cl-OHPA, strain PCE1 shows activity towards Cl-OHPA and various DCPs (2,3-, 2,4-, 2,5-, and 2,6-) (van de Pas et al. 2001b). Growth of strain PCE1 on Cl-OHPA induces the production of a 48-kDa chlorophenol reductive dehalogenase which does not dehalogenate PCE or TCE, while the PCE reductive dehalogenase does not dehalogenate chlorophenols, indicating that two different enzymes are produced for these two reactions (van de Pas et al. 2001b).

A relatively recent addition to the group of reductive dehalogenases from the *Desulfitobacterium* genus was identified in *Desulfitobacterium* sp. strain Y51. Similar to other PCE/TCE reductive dehalogenases, the purified 58-kDa PCE reductive dehalogenase (PceA) is a corrinoid-containing protein (Suyama et al. 2002). Unlike other reductive dehalogenases, however, the mature enzyme from strain Y51 was localized to the periplasm and not in association with the inner membrane. The unprocessed enzyme is localized to the cytoplasm and has a molecular weight of 67 kDa (Suyama et al. 2002). In comparison, PCE dehalogenase from *D. multivorans* has been purified from the cytosol, while PCE dehalogenases from strains *Desulfitobacterium* sp. PCE-S, *D. restrictus*, and *D. ethenogenes* have been found to be associated with the membrane

fraction. The mature enzyme from strain Y51 is able to dehalogenate PCE, TCE, hexa- and penta-chloroethanes (Suyama et al. 2002). Lower chlorinated ethenes and ethanes (trichloroethanes) and chlorobenzoates were not dehalogenated. Suyana et al. (2002) suggested growth of strain Y51 on PCE or TCE as the sole energy-generating electron acceptor was a result of dehalorespiration. Though, no mechanism was proposed to account for the fact that this enzyme is present on the periplasmic side of the inner membrane and how its activity can result in energy conservation. This differs from other reductive dehalogenases which are integral membrane proteins or membrane-associated but present on the cytoplasmic side of the inner membrane. Orientation of the dehalogenase on the cytoplasmic side of the inner membrane likely results in the development of an electrochemical gradient via a scalar mechanism, which does not involve proton translocation from one side of the membrane to the other to generate a PMF. Thus, for dehalorespiration to occur, strain Y51 would potentially require a mechanism of translocating H^+ to establish a chemiosmotic gradient due to its reductive dehalogenase being present in the periplasm.

Other than species of *Desulfitobacterium*, PCE reductive dehalogenases have been described in other bacterial species. A description of three examples found in species of *Dehalobacter*, *Dehalospirillum* and *Dehalococcoides* follows.

Dehalobacter restrictus was first isolated from a Rhine river sediment mixed with granular sludge (Holliger et al. 1998). Similar to those of *Desulfitobacterium* sp., the enzymes from *D. restrictus* has a narrow substrate range and can dehalogenate PCE and TCE only. The *D. restrictus* PCE reductive dehalogenase is a 60-kDa membrane-bound protein containing a cobaltous corrinoid and two Fe_4S_4 clusters. The cobalamin in the

enzyme has a relatively high redox potential, as compared to other microbial corrinoid enzymes, resulting in an easily reducible cobalamin, i.e. it readily accepts electrons (Schumacher et al. 1997). Likely, other PCE reductive dehalogenases with similar corrinoid factors may also be easily reduced. The iron sulfur clusters were found to be of the Fe₄-S₄ cubane type and had a relatively low redox potential (Schumacher et al. 1997). Thus, the FeS clusters were considered to be electron shuttles rather than locations of electron storage. Although the physiologically relevant electron donor has not been identified, the proposed electron transfer scheme within the *D. restrictus* reductive dehalogenase is from electron donor to cubane 2 to cubane 1 (the iron sulfur clusters) to the cobalamin and finally to the electron acceptor, PCE (Schumacher et al. 1997). Biochemical analyses of the *D. restrictus* reductive dehalogenase have yet to be published.

Dehalospirillum multivorans, isolated from activated sludge, is capable of dechlorinating PCE to *cis*-1,2-DCE via TCE with the production of energy (Scholzmuramatsu et al. 1995). The monomeric enzyme with a molecular weight of ~57.5 kDa has been purified (Neumann et al. 1996). This reductive dehalogenase is similar to others in several respects, including the presence of FeS clusters, a corrinoid factor and oxygen sensitivity (Neumann et al. 1996). In contrast to other reductive dehalogenases from anaerobic bacteria, the PCE reductive dehalogenase from *D. multivorans* was localized to the cytoplasm (Miller et al. 1996). The substrate range of the purified enzyme is very narrow, being active with PCE, TCE, various chloropropenes and, to a lesser extent, tetraiodoethene (Neumann et al. 1996, Neumann et al. 2002). Interestingly, the purified enzyme can dechlorinate chloropropenes, although *D. multivorans* is unable to grow on

these compounds as electron acceptors (Trescher 1998). The enzyme is not active against a variety of dichloroethenes, trichloroethanes, trichloroacetone, hexachloroethane, 3-CB, 4-chlorophenol, and 3,4-dichlorobenzoate (Neumann et al. 1996).

Identification of a non-dechlorinating strain of *D. multivorans* (strain N) has helped in understanding the essential role of the corrinoid factor during reductive dehalogenation. The non-dechlorinating strain N was isolated from the same site as the dechlorinating strain K. Strain N possesses the reductive dehalogenase gene sequence *pceA* and the anchor protein gene sequence *pceB*, although *pceA* is transcribed and translated to a much lesser extent than in strain K (Siebert et al. 2002). Furthermore, the expressed reductive dehalogenase in strain N lacked the corrinoid factor found in the strain K dehalogenase and this was believed to be responsible for the inability of the strain N enzyme to dechlorinate PCE and TCE (Siebert et al. 2002). Siebert et al. (2002) suggest that the corrinoid factor may have a regulatory role in the transcription and/or translation of *pceA*. The corrinoid factor from *D. multivorans* contains a cobalt center; however, its characteristics are quite different from the more common cobalt-containing corrinoid vitamin B₁₂ (Neumann et al. 2002). The precise nature of the *D. multivorans* corrinoid factor remains to be elucidated.

A common feature of the reductive dehalogenases from *Desulfitobacterium*, *Dehalobacter* and *Dehalospirillum* strains is that the enzymes catalyze the dechlorination of PCE to TCE and then to *cis*-1,2-DCE, which is toxic. Accumulation of *cis*-1,2-DCE inhibits the dehalogenases through an as yet unknown mechanism; thus, another enzyme or microorganism is required to eliminate this toxic metabolite.

Dehalococcoides ethanogenes 195 was the first microorganism found to contain a

novel catabolic pathway capable of degrading PCE and TCE to ethene, an environmentally harmless compound (Maymo-Gatell et al. 1997). *Dehalococcoides ethenogenes* 195 utilizes the chloroalkenes for growth via dehalorespiration. This microorganism possesses two enzymes with Co(I) corrinoid cofactor, a PCE reductive dehalogenase and a TCE reductive dehalogenase (Magnuson et al. 1998). PCE reductive dehalogenase, believed to be constitutively expressed, catalyzes the transformation of PCE to TCE (Magnuson et al. 1998, Maymo-Gatell et al. 1999). TCE reductive dehalogenase, a 57-kDa peripheral membrane protein, catalyzes the degradation of TCE to ethene, via DCE and VC (Magnuson et al. 1998, Maymo-Gatell et al. 1999). TCE reductive dehalogenase has been shown to dechlorinate other haloalkenes and haloalkanes; however, it prefers two carbon compounds containing two or three halogens, preferably on non-adjacent carbons (Magnuson et al. 2000).

1.5.4.1 Molecular characterization

Sequence analysis of the *pceA* gene products from *D. spirillum* and *Desulfitobacterium* sp. strain Y51 revealed traits common to other reductive dehalogenases and provided clues to some of their characteristics. These include the presence of the twin Arg translocation consensus sequence (RRXFXK), a ferredoxin-like cluster (CXXCXXCXXXCP) and a second iron-sulfur cluster (GXXCXXCXXCS). Both dehalogenases lack the consensus sequence for a corrinoid-binding site (DXHXXG). For both organisms, the *pceA* gene is upstream of the *pceB* (Suyama et al. 2002), which codes for the small hydrophobic protein thought to anchor PceA to the membrane. In *D. spirillum*, the C-terminus of *pceA* and the N-terminus of *pceB* overlap by four basepairs,

indicating that the two proteins are co-transcribed (Neumann et al. 1998). In strain Y51, two ORFs upstream of the *pceA* gene were identified. One (ORF1) has no similarity to sequences present in the database analyzed, while the second ORF (ORF0) was partially sequenced at the C-terminus and it showed similarity to several transposases (Suyama et al. 2002).

The *tceA* gene encoding TCE reductive dehalogenase from *D. ethenogenes* 195 has been cloned and sequenced, along with the *tceB* gene, which is downstream of *tceA*. The two genes are believed to be co-transcribed (Magnuson et al. 2000). The 10-kDa TceB contains three membrane-spanning regions and is believed to be the membrane anchor for TceA. TceA is believed to be a new subclass of oxidoreductase containing cobalamin and iron-sulfur clusters as co-factors (Magnuson et al. 2000). The two other proteins from *Dehalospirillum multivorans* and *Desulfitobacterium dehalogenans* that have been characterized have a similar pattern in the placement of the genes, i.e. the gene for the catabolic enzyme (*pceA* and *cprA*) is followed downstream by a smaller gene (*pceB* and *cprB*) coding for the small integral membrane protein (van de Pas et al. 1999). PceA from *D. multivorans* and TceA from *D. ethenogenes* 195 share a low level of sequence homology despite both being able to dehalogenate TCE (Magnuson et al. 2000). The primary sequence of TceA contains two iron-sulfur clusters of the Fe₄-S₄ variety, while the sequences of PceA and CprA contain one Fe₄-S₄ and one Fe₄-S₃ cluster (Magnuson et al. 2000). However, the role of the different iron sulfur clusters has not been examined.

1.6 Reductive Dehalogenases Involved in Carbon Metabolism

Carbon catabolism is the enzymatic breakdown of larger organic compounds into smaller compounds. This vital process is a major source of energy for many microorganisms; however, most of the enzymes involved in carbon metabolism are unable to degrade halogenated organic compounds because of interference by the halogen groups. The metabolic breakdown of many halogenated organic compounds can only occur once the halogen atoms are removed, a role fulfilled by dehalogenases. Acting either alone, or as part of a multiple enzyme dehalogenation pathway, reductive dehalogenases remove the halogen groups from a wide assortment of halogenated organic compounds. The dehalogenated organic compound(s) may then be mineralized to carbon dioxide and water through a microorganism's carbon metabolism pathway. Reductive dehalogenases that aid carbon metabolism have been observed in facultative anaerobic organisms (Egland et al. 2001, McGrath and Harfoot 1997), and in several aerobic bacteria (Crawford and Ederer 1999, Fetzner 1998, Haggblom et al. 1988, Haggblom et al. 1989, Kumari et al. 2002, Leung et al. 1997, Masai et al. 1993, Miyauchi et al. 1998, Romanov and Hausinger 1996, Saber and Crawford 1985, Schenk et al. 1989, Uotila et al. 1991, Xun et al. 1992b). What makes these enzymes particularly interesting to study in aerobic bacteria is that they are present in microorganisms that require oxygen to grow and divide, and yet, the presence of that same oxygen adversely affects the ability of the reductive dehalogenases to function properly.

Metabolism of a bromoalkane and various chloroalkanes by the facultative anaerobes *Rhodospirillum rubrum*, *Rhodospirillum photometricum*, and *Rhodopseudomonas palustris* has been observed (Egland et al. 2001, McGrath and

Harfoot 1997). The mechanism for 3-chlorobenzoate (3-CB) degradation has been described for *Rhodopseudomonas palustris*. 3-CB is metabolized first via conversion to 3-CB-CoA, which is dechlorinated by an unknown reductive dehalogenase to benzoyl-CoA, which can then be used as a carbon source (Egland et al. 2001). Other examples of aerobic bacteria that generate CoA thioesters in the reductive degradation of chlorinated benzoates include *Corynebacterium sepedonicum* KZ-4 and Coryneform bacterium strain NTB-1 (Romanov and Hausinger 1996).

An extensively studied group of reductive dehalogenases in the catabolic pathways of aerobic microorganisms is the glutathione transferases (GSTs). GSTs are found in both prokaryotes and eukaryotes that catalyze the conjugation of glutathione to an electrophilic xenobiotic and/or endogenous compound. Presently, this group of enzymes is divided into three superfamilies: the canonical GSTs, the membrane-bound microsomal GSTs, and the fosfomycin-resistance GSTs (Armstrong 1998). Among these, the most relevant to reductive dehalogenation are the canonical or soluble GSTs. This group is divided into several classes that include the alpha (α), beta (β), delta (δ), epsilon (ϵ), kappa (κ), mu (μ), pi (π), theta (θ), sigma (σ), zeta (ζ), and omega (ω) subdivisions. The classification scheme of the soluble GSTs may, however, be subject to change with new characteristics being discovered in existing members and additional members being identified. Several soluble bacterial GSTs catalyze the removal of chlorine atoms from organic substrates as part of catabolic pathways. The best-known examples of these pathways include the biodegradation of dichloromethane and the recalcitrant xenobiotics PCP and lindane. Two well-studied bacterial soluble GSTs are dichloromethane (DCM) dehalogenase and tetrachlorohydroquinone (TCHQ) reductive dehalogenase (see section

1.4).

1.6.1 Dichloromethane (DCM) dehalogenase

The DCM dehalogenase is an example of a solitary reductive dehalogenase involved in carbon metabolism. This enzyme dechlorinates DCM to formaldehyde, which is funneled into the carbon and energy metabolic pathways of methylotrophic bacteria (Leisinger et al. 1994, Leisinger and Braus-Stromeyer 1995). This enzyme has been reviewed previously (Copley 1998, Leisinger et al. 1994, Leisinger and Braus-Stromeyer 1995, Vuilleumier 1997a, Vuilleumier and Pagni 2002). Here, we provide an overview of some of the key points relating to this enzyme.

1.6.1.1 Enzyme characteristics

Most studies on DCM dehalogenases have been done on enzymes from *Methylobacterium dichloromethanicum* DM4 (previously *Methylobacterium* sp. strain DM4 (La Roche and Leisinger 1990)) and *Methylophilus* sp. strain DM11 (Bader and Leisinger 1994, Evans et al. 2000, Gisi et al. 1998, Gisi et al. 1999, Kayser et al. 2000, Kayser and Vuilleumier 2001, Kayser et al. 2002, La Roche and Leisinger 1990, La Roche and Leisinger 1991, Marsh and Ferguson 1997, Scholtz et al. 1988, Vuilleumier and Leisinger 1996, Wheeler et al. 2001). The primary sequences of these enzymes share 56% identity. They catalyze the dechlorination of DCM to formaldehyde in a two-step process (Figure 1.3). In the first step, DCM dehalogenase catalyzes the conjugation of DCM with GSH, forming an unstable S-chloromethylglutathione with the loss of a

chlorine atom. In the second step, the conjugated product spontaneously reacts with water to form *S*-hydroxymethylglutathione with the loss of a second chlorine atom (Wheeler et al. 2001). *S*-Hydroxymethylglutathione is further converted spontaneously to formaldehyde and GSH (Wheeler et al. 2001).

Figure 1.7 Dechlorination of dichloromethane to formaldehyde by dichloromethane dehalogenase with glutathione (GSH) as a cofactor. Two reaction intermediates are formed: *S*-chloromethyl-glutathione and *S*-hydroxymethyl-glutathione (Wheeler et al. 2001).

The two dehalogenases from DM4 and DM11 differ significantly in kinetic parameters and dehalogenase expression levels. For example, the DM4 DCM dehalogenase exhibits approximately six-fold lower $V_{\max}$ and K_m values than the DM11 dehalogenase. Thus, the DM4 DCM dehalogenase works more slowly, but it has a greater affinity for DCM than the DM11 dehalogenase. DCM dehalogenase can be expressed to a greater extent by strain DM4 (15% of the total cell protein) relative to strain DM11 (7% of the total cell protein) (Scholtz et al. 1988). These differences affect the conditions under which each microorganism functions best. *Methylobacterium*

dichloromethanicum DM4 grows and divides optimally under continuous culture and DCM-limited conditions (Gisi et al. 1998), while *Methylophilus* sp. strain DM11 grows best in batch culture with an excess of DCM (Gisi et al. 1998). These differences were postulated to be adaptive responses to conditions found in the natural environments from which each of the bacteria was isolated (Gisi et al. 1998). The above differences have been used to divide the DCM dehalogenases into two groups represented by the DM4 enzyme (Group A) and DM11 enzyme (Group B), respectively.

An interesting secondary reaction arising from the action of DCM dehalogenase is the formation of DNA adducts (Kayser and Vuilleumier 2001). One might reason that formaldehyde formed from DCM degradation may have mutagenic effects upon the degrading microorganism; however, formaldehyde is not mutagenic to *M. dichloromethanicum* DM4 (Wheeler et al. 2001) and this strain is able to grow on DCM (Kayser et al. 2000). Subsequent studies found that it is the *S*-chloromethylglutathione that is problematic. In experiments using either ^{14}C -DCM or ^{35}S -GSH, the radiolabeled *S*-chloromethylglutathione produced by DCM dehalogenase formed adducts with DNA resulting in DNA breakage (Kayser and Vuilleumier 2001). Subsequent experiments with whole cells of *Methylobacterium dichloromethanicum* DM4 and its *polA* mutant (a DNA polymerase deficient mutant) verified that DCM degradation results in DNA damage (Kayser and Vuilleumier 2001). Although both strains produce similar levels of DCM dehalogenase, the *polA* mutant showed a greater frequency of DNA strand breaks (Kayser and Vuilleumier 2001). The results suggested that DNA polymerase I is needed to repair DNA breaks when *Methylobacterium dichloromethanicum* DM4 is grown on DCM as a sole carbon and energy source (Kayser et al. 2000, Kayser and Vuilleumier

2001).

1.6.1.2 Molecular characterization

DCM dehalogenase is an inducible enzyme encoded by the *dcmA* gene (La Roche and Leisinger 1991). In strain DM4, *dcmA* is regulated by *dcmR*, a 30 kDa putative DCM-specific repressor that prevents transcription of *dcmA* (La Roche and Leisinger 1991). In addition, in DM4 and other DM4-like methylotrophic bacteria the *dcmA* and *dcmR* genes have been localized to a 4.2 kb *Bam*HI fragment (Schmid-Appert et al. 1997). The fragment is flanked by insertional sequence elements suggesting the DCM catabolic genes may, potentially, be transferred horizontally by transposition (Schmid-Appert et al. 1997).

An analysis of DCM-degrading bacteria retrieved from enrichment cultures and sludge from wastewater treatment facilities has shown similarity to DM4 dehalogenase based on cross-reactivity with polyclonal antibodies raised against the DCM dehalogenase from DM4 strain (Kohler-Staub et al. 1986) and *dcmA* hybridization tests (Scholtz et al. 1988). A more detailed examination of *dcmA* gene sequences has revealed slight variations among different DCM-degrading isolates, mainly as single nucleotide substitutions (Vuilleumier et al. 2001). From fifteen DCM-degrading bacterial isolates, eight different *dcmA* gene sequences were found, and this translates into seven different DcmA amino acid sequences (Vuilleumier et al. 2001). A strain isolated from chlorinated aliphatic hydrocarbon-contaminated sludge in the Netherlands (GJ21) had the greatest number of substitutions, with fifteen nucleotide substitutions corresponding to nine amino acid changes (Vuilleumier et al. 2001). No significant differences in

dehalogenase activity were noted for the seven different DCM dehalogenases when compared to the DCM dehalogenase from strain DM4 (Vuilleumier et al. 2001). These results indicate the sequence and enzymatic properties of DCM-degrading aerobic methylotrophic bacteria are well conserved (Vuilleumier et al. 2001).

1.6.1.3 Structural characterization

In strain DM4, *dcmA* encodes a 287 amino acid protein with a predicted molecular weight of 37.4 kDa (La Roche and Leisinger 1990). In contrast, the *dcmA* gene in strain DM11 encodes a 267 amino acid protein with a predicted molecular weight of 31.2 kDa (Bader and Leisinger 1994). The DCM dehalogenases have been placed in the GST superfamily of enzymes based on several lines of evidence. Site-directed mutagenesis studies have identified an essential Ser in the N-terminal cofactor-binding site, typical of the theta class GSTs (Vuilleumier and Leisinger 1996). A subsequent study has identified another N-terminal amino acid (Ala27 for the DM4 enzyme and Val18 for the DM11 enzyme) important for glutathione binding (Vuilleumier et al. 1997b). Mutations of Ala27 in DM4 and Val18 in DM11 are believed to cause conformational changes in the DCM dehalogenase resulting in decreased affinity for glutathione (Vuilleumier et al. 1997b). This requirement for glutathione supports the view that the dichloromethane dehalogenases are GSTs (Vuilleumier et al. 1997b). Lastly, a 3-D structural homology model of the DM11 DCM dehalogenase predicted an affiliation with the glutathione binding site of the theta class GSTs (Marsh and Ferguson 1997).

1.7 Concluding Remarks

Bacterial reductive dehalogenases are a major class of dehalogenases that perform the crucial function of removing halogen substituents from many chlorinated organic compounds. This activity initiates the biodegradation of many recalcitrant and toxic xenobiotic compounds, such as PCP, resulting in their detoxification and in some instances their ultimate mineralization. In this chapter, many of the general biochemical and molecular characteristics of bacterial reductive dehalogenases are presented. Although many reductive dehalogenases have been purified, their genes cloned, sequenced and characterized, much remains unknown about these enzymes.

To date, none of the reductive dehalogenases have been crystallized, and therefore, detailed structural information is not available. Biochemical and genetic studies have provided some insight into the structure and function of the reductive dehalogenases. Many anaerobic microorganisms capable of reductive dehalogenation of chlorophenol, TCE, and PCE possess reductive dehalogenases that are based on a similar foundation, involving similar redox centers, enzyme transport processes and enzyme maturation. With regard to reductive dehalogenases from aerobic microorganisms, only DCM dehalogenase has been studied in some detail. More research is needed to increase our understanding of how reductive dehalogenases work. This is particularly evident for the reductive dehalogenases found in aerobic microorganisms.

1.8 Thesis Objectives and Hypotheses

The primary objective of this thesis is to examine the structure-function relationships of a reductive dehalogenase from an aerobic microorganism, namely the TCHQ reductive dehalogenase (PcpC) from *Sphingomonas* sp. UG30. *Sphingomonas* sp. UG30, a strain isolated by University of Guelph researchers from PCP-contaminated soil, dechlorinates and mineralizes high levels of PCP (Leung et al. 1997). Further, *Sphingomonas* sp. UG30 possesses a PCP degradation pathway that includes three principal initial enzymes homologous to those of the well-studied *Sphingobium chlorophenolicum* ATCC 39723 (Cassidy et al. 1999, Leung et al. 1997, Leung et al. 1999). The three enzymes acting sequentially are: PCP-4-monoxygenase (PcpB) (Lange et al. 1996, Xun and Orser 1991), TCHQ reductive dehalogenase (PcpC) (McCarthy et al. 1996, Orser et al. 1993, Xun et al. 1992b) and dichlorohydroquinone (DCHQ) dioxygenase (PcpA) (Xu et al. 1999, Xun et al. 1999).

Currently, studies of UG30 have concentrated on the ability of whole cells to degrade chlorophenols and nitrophenols under various conditions (Alber et al. 2000, Cassidy et al. 1997, Cassidy et al. 1997, Cassidy et al. 1999, Zablotowicz et al. 1999). In addition, experiments have examined the role of PcpB from UG30, with regard to the degradation of nitrophenolic compounds (Leung et al. 1999). Beyond the determination of the presence of the *pcpC* gene, however, little else has been studied with the PcpC from UG30. In several *Sphingobium* strains genes of PcpC have been isolated and examined. Differences have been found in the genes themselves and two distinct classes of PcpC enzymes have been observed using Southern analysis using a 0.7-kb *EcoR*I fragment of

the *S. chlorophenolicum* ATCC 39723 *pcpC* gene as a probe (Crawford and Ederer 1999). The *S. chlorophenolicum* strains ATCC 39723 and RA2 formed one group and *S. chlorophenolicum* strains SR3 and ATCC 33970 formed another (Crawford and Ederer 1999, Ederer et al. 1997). With the high homology to ATCC 39723, the UG30 PcpC would likely group with it. Thus, for comparison, the ATCC 39723 PcpC would provide a good base from which to work.

Some studies have already been undertaken on the highly homologous PcpC from *S. chlorophenolicum* ATCC 39723 examining some of its functional and kinetic parameters (Anandarajah et al. 2000, Orser et al. 1993, Xun 1992a, Xun et al. 1992b). In addition, aspects of its reaction mechanism have been elucidated (Kiefer and Copley 2002a, Kiefer et al. 2002b, McCarthy et al. 1996); however, many questions remain unanswered, or are unanswerable, using the *S. chlorophenolicum* PcpC as a model. This results from the severe substrate inhibition experienced by the *S. chlorophenolicum* PcpC, which has reduced the extent of kinetic and mutational studies that can be carried out on the wildtype enzyme (Anandarajah et al. 2000, Kiefer and Copley 2002a, Kiefer et al. 2002b). For example, only the N-terminal Cys has been examined in the ATCC 39723 PcpC, but there are several other N-terminal amino acids (including Tyr and Ser residues) that have the potential to be involved in the reaction mechanism and remain unexamined. These are areas where studies of the PcpC from *Sphingomonas* sp. UG30 can further develop our understanding of this class of enzymes.

The initial objective of my work is to characterize the wildtype PcpC enzyme from *Sphingomonas* sp. UG30. Although the PcpC enzyme is constitutively expressed within *Sphingomonas* sp. UG30 (Cassidy et al. 1999), as in other bacteria expressing this

protein, its low expression levels within the cell make it difficult to isolate directly from *Sphingomonas* sp. UG30. By using recombinant techniques to clone the *pcpC* gene into a vector for expression in *E. coli* large quantities of the enzyme can be produced and purified for further characterization. This is reasonable for an enzyme such as PcpC since it originates from a microorganism and does not appear to undergo any further post-translational modification following expression. In addition, the PcpC from *S. chlorophenolicum* has been previously cloned and an active enzyme was produced (McCarthy et al. 1996, Orser et al. 1993). With a source of recombinant enzyme having been made available, an examination of its functional, kinetic, and structural properties can be undertaken. With this information, comparisons to the highly homologous *S. chlorophenolicum* PcpC can also be studied. Based upon their high degree of amino acid identity (94%) and their presence in a degradative pathway capable of specifically breaking down PCP, both PcpC enzymes should possess similar functional and kinetic parameters.

The second objective of my research is to examine amino acids critical for the catalysis of TCHQ by PcpC. This is important for several reasons. With regard to PcpC directly, as has already been mentioned, no amino acids other than the N-terminal Cys13 have been modified or mutated. In addition, Ser12 of the ATCC PcpC has been postulated to be involved in catalysis, however, this has not been tested experimentally. With regard to the GSTs, examination of critical amino acids present at the N-terminus of the enzyme can aid in defining to which class a particular enzyme belongs. With regard to other bacterial GSTs site-directed mutagenesis studies may aid in identifying commonalities between the bacterial GSTs that are placed in various GST classes,

including the Beta, Theta, and Zeta classes.

This work focuses on two groups of amino acids: the N-terminal amino acids present in the glutathione-binding site of the GSTs that include Tyr, Ser, and Cys residues; and His and Lys residues. The GST class specific N-terminal Tyr (present in the Alpha, Mu, and Pi GST classes) or Ser (present in the Theta and Zeta GST classes) have been found to typically aid in stabilizing the glutathione thiolate ion within the glutathione-binding site (Sheehan et al. 2001). One exception is the Beta class, although N-terminal Tyr and Ser residues are present neither is involved in the catalytic mechanism (Casalone et al. 1998, Nishida et al. 1994). The N-terminal Cys has been shown to play different roles in the GSTs of various classes, ranging from simply aiding in the orientation of glutathione in the active site (Board et al. 2003, Polekhina et al. 2001) to forming a direct disulfide bond with glutathione (Rossjohn et al. 1998). In the *S. chlorophenolicum* ATCC 39723 PcpC, Cys13 has been shown to play a critical role in proper product formation (McCarthy et al. 1996, McCarthy et al. 1997). The relevant working hypothesis is that mutation studies will confirm the placement of the UG30 PcpC in the zeta class of GST enzyme family and that N-terminal Ser12 and Cys14 residues will be essential for activity.

The second group of mutations to be examined involves His and Lys residues. In several classes of GST, His and/or Lys residues have been shown to participate in catalysis or aid in orienting the substrate or co-substrate within the active site. Sequence comparison of the UG30 PcpC with other bacterial GSTs revealed conservation of one His residue and one Lys residue each found to be important for activity in the GSTs from *P. mirabilis* and *E. coli*. In *P. mirabilis* His106 and Lys107 help orient GSH in the

glutathione-binding site (Allocati et al. 2000). In *E. coli* His106 is proposed to stabilize the negatively charged thiolate ion within the glutathione-binding site (Nishida et al. 1998), as has been shown for a human Mu class GST. In addition, studies of the human and *A. thaliana* Zeta class GSTs have not definitively identified an N-terminal amino acid residue that stabilizes the glutathione thiolate ion within the active site (Board et al. 2003, Polekhina et al. 2001, Thom et al. 2001). Thus, there is the potential for His and/or Lys residues to play a role in the Zeta class GSTs.

The UG30 PcpC contains four His residues and a Lys residue, Lys114, which corresponds to Lys107 in the *P. mirabilis* GST. Chemical modification experiments using the His-specific modifier diethyl pyrocarbonate (DEPC) will provide an initial determination of the impact of His residues in the UG30 PcpC. Subsequently, site directed mutagenesis of each of the four His residues present in PcpC as well as Lys114, will aid to assess their individual and specific roles in UG30 PcpC activity. It is also hypothesized that His113 and Lys114 residues may be important for activity in PcpC with roles principally involved in orientation of GSH in the active site.

The experiments are presented as published manuscripts or manuscripts prepared for publication in the chapters that follow and the relevance of the results obtained with regard to the structure-function relationships of bacterial reductive dehalogenases is discussed.

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CHAPTER TWO

Cloning, Expression, and Characterization of Tetrachlorohydroquinone Reductive Dehalogenase from *Sphingomonas* sp. UG30^b

2.1 Introduction

Pentachlorophenol (PCP), a highly chlorinated xenobiotic compound, is used primarily as a fungicide for wood preservation (McAllister et al. 1996). Within the last 25 years, the use of PCP has been restricted or banned in many countries because of its persistence in soil and water. As a suspected human carcinogen, PCP poses a serious risk to environmental and human health. Despite being present in the environment for only about 66 years (Cline et al. 1989) with no known natural analogs, a number of microorganisms have evolved mechanisms to tolerate and/or degrade PCP (Apajalahti and Salkinoja-Salonen 1987, Haggblom et al. 1988, Haggblom et al. 1989, Leung et al. 1997, Saber and Crawford 1985, Uotila et al. 1991).

Sphingomonas sp. UG30, a strain isolated by University of Guelph researchers from PCP-contaminated soil, is able to dechlorinate and mineralize high levels of PCP (Leung et al. 1997). The strain is capable of adapting to higher PCP levels by varying its outer membrane composition (Lohmeier-Vogel et al. 2001). Further, *Sphingomonas* sp. UG30 possesses an enzymatic pathway, capable of PCP degradation, involving three enzymes homologous to those of the well-studied *Sphingobium chlorophenolicum* ATCC 39723 (previously *Sphingomonas chlorophenolica* ATCC 39723 (Takeuchi et al. 2001)).

^b Portions of this chapter have been published in Habash, M.B., L.A. Beaudette, M.B. Cassidy, K.T. Leung, T.A. Hoang, H.J. Vogel, J.T. Trevors, and H. Lee. 2002. Biochemical and Biophysical Research Communications 299:634-640.

The three enzymes acting sequentially are: PCP-4-monooxygenase (PcpB) (Lange et al. 1996, Xun and Orser 1991), tetrachlorohydroquinone reductive dehalogenase (PcpC) (McCarthy et al. 1996, Orser et al. 1993, Xun et al. 1992) and dichlorohydroquinone dioxygenase (PcpA) (Xu et al. 1999). The NADPH-dependent PcpB carries out an oxygenolytic attack on PCP, replacing the chlorine at the para position with an -OH group to yield tetrachloro-*p*-hydroquinone or TCHQ (Lange et al. 1996, Xun and Orser 1991). The glutathione-dependent PcpC reductively removes 2 chlorines from TCHQ to form first trichlorohydroquinone (TriCHQ) and then dichlorohydroquinone (DCHQ) (McCarthy et al. 1996, Orser et al. 1993, Xun et al. 1992). PcpA then cleaves the aromatic ring of DCHQ to yield a product that can be mineralized to CO₂ and H₂O (Ohtsubo et al. 1999, Xu et al. 1999, Xun et al. 1999).

PcpC is a member of the glutathione transferases (GSTs) that are widely found in both prokaryotes and eukaryotes. Eight classes of GSTs have been proposed. PcpC was initially placed in the Theta class (McCarthy et al. 1996, Xun et al. 1992) and more recently in the Zeta class (Anandarajah et al. 2000, Board et al. 1997) of the GST superfamily. The primary role of GSTs in many organisms is to aid in detoxification processes (Sheehan et al. 2001, Vuilleumier 1997). The incorporation of GSTs into enzymatic pathways of microorganisms able to degrade recently introduced xenobiotic compounds such as PCP, coupled with their broad substrate range suggests some GSTs may be recently evolved enzymes. For example, along with its dehalogenase activity, PcpC from *S. chlorophenolicum* ATCC 39723 has been shown to exhibit isomerase activity (Anandarajah et al. 2000) similar to that of a human Zeta class GST, maleyl acetoacetate isomerase (Board et al. 1997). This interesting link has provided

information on the origin of PcpC and may aid in understanding its mechanism of action.

Although detailed structural studies have yet to be performed on PcpC, its reaction mechanism has been studied for the *S. chlorophenolicum* ATCC 39723 PcpC (Anandarajah et al. 2000, Kiefer and Copley 2002, Kiefer et al. 2002, McCarthy et al. 1996, Xun 1992, Xun et al. 1992). In this study, we cloned the *pcpC* gene from *Sphingomonas* sp. UG30 and expressed it in *E. coli*. Even though the UG30 PcpC exhibits a high degree of similarity to the *S. chlorophenolicum* PcpC, it possesses some significant differences. The UG30 PcpC exhibits higher pH and temperature optima than does the *S. chlorophenolicum* PcpC. In addition, the *S. chlorophenolicum* PcpC experiences severe inhibition by the substrate TCHQ, thus necessitating the use of a Cys13Ser mutant enzyme for kinetic and mechanistic studies (Anandarajah et al. 2000, Kiefer and Copley 2002, Kiefer et al. 2002). In contrast, we did not find any evidence of inhibition of the UG30 PcpC by TCHQ. This may allow a more detailed examination of the catalytic mechanism using the wild type enzyme.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Plasmids

The *pcpC* gene was cloned from the genomic DNA of *Sphingomonas* sp. UG30 (described below). UG30 was grown at 30°C in a minimal medium supplemented with glutamate and shaken at 200 rpm as previously described (Leung et al. 1997). The genomic DNA fragment carrying the *pcpC* gene was initially cloned into pBluescript and propagated in *Escherichia coli* JM109. Plasmid pTXB1 from the Impact-CN kit (New

England BioLabs, Mississauga, ON, Canada) was used as a protein expression vector in *Escherichia coli* ER2566. In pTXB1, the C-terminus of the insert is fused to a chitin-binding intein sequence. Upon expression, the insert-intein fusion protein can be affinity purified on a chitin column. In the presence of dithiothreitol (DTT), the intein tag undergoes specific self-cleavage to release the insert protein from the intein tag. After cleavage, there is no vector-derived extra amino acid(s) on the recombinant insert protein. ER2566 cells were grown in Luria broth (LB) at 37°C and shaken at 200 rpm or grown on an LB agar plate supplemented with ampicillin to a final concentration of 100 µg/mL as needed. For long-term storage, stocks of *E. coli* ER2566 and *Sphingomonas* sp. UG30 were prepared in 50% v/v glycerol and stored at -20°C.

2.2.2 Cloning of *pcpC*

Genomic DNA was isolated from *Sphingomonas* sp. UG30 using the phenol/chloroform method (Ausubel et al. 1989), restricted with *Hind*III and the fragments run on a 1% (w/v) agarose gel. The fragment containing the *pcpC* gene encoding TCHQ reductive dehalogenase was identified using a DIG-labeled probe. The probe was synthesized by PCR using genomic DNA from *Sphingobium chlorophenolicum* ATCC 39723 as the template and primers targeting the entire coding sequence of the *S. chlorophenolicum pcpC* gene, which is available at the GenBank under Accession number M98559. The 8 kb *pcpC*-positive fragment from the UG30 genomic DNA was isolated, further restricted and probed. The *pcpC* gene was ultimately localized to a 2.5-kb *Sma*I-*Xho*II fragment. This fragment was cloned into pBluescript, and transformed into *E. coli* JM109. The insert contained the full coding sequence of *pcpC*, as

ascertained by the ability of the *S. chlorophenolicum* *pcpC* primers to amplify a fragment of similar size (about 750 bp). The plasmid bearing the UG30 *pcpC* gene was isolated from *E. coli* JM109 and the insert sequenced in both directions using an Applied Biosystems Model 377 automated sequencer at the Laboratory Services Division (LSD), University of Guelph. The complete sequence of this fragment has been deposited at the GenBank under Accession No. AY057901.

2.2.3 Expression and purification of PcpC protein

For expression, the UG30 *pcpC* gene was subcloned into pTXB1 by PCR. Two primers were designed based on the sequence information to amplify the entire coding region of this gene. The primers also incorporated *NdeI* (PcpcNdeI: 5'-TTCCGGGAATTCCCATATGTCTGAAGTCAGTCTCTATA – 3') and *SapI* (PcpcSapI: 5' – GGTGGTTGCTCTTCCGCAGATGCCGCCCTTCCAGTT – 3') restriction sites (underlined in the above sequences) at their respective 5'-ends to facilitate directional cloning into pTXB1. The lone PCR product of the expected size (~750 bp) was gel purified, digested with *NdeI* and *SapI*, and ligated into the *NdeI*-*SapI* sites in pTXB1 to yield the plasmid pTXB1PcpC. The ligation product was initially transformed into competent *E. coli* JM109 cells for maintenance of the plasmid. One clone was randomly selected and the correct insert size and orientation in the recombinant plasmid was confirmed by PCR using insert-specific and vector-specific primers. The sequence of the insert (*pcpC*) in pTXB1PcpC was determined in both directions using vector-specific primers. After sequence verification, pTXB1PcpC was isolated from *E. coli* JM109 and transformed into competent *E. coli* ER2566 cells for expression of the PcpC/intein tag

fusion protein. One clone was randomly selected, the plasmid isolated and the correct insert was further confirmed by DNA sequencing in one direction. Frozen stocks (in 50% v/v glycerol) of ER2566 cells carrying the pTXB1PcpC plasmid were prepared and stored at -20°C for subsequent expression and purification of the wild type PcpC protein.

Protein expression was performed as described for the IMPACT CN- kit with some modifications (New England Biolabs). Fifty (50) microlitres of *E. coli* ER2566 (pTXB1PcpC) frozen stock were inoculated into 50 mL LB broth containing 100 $\mu\text{g/mL}$ ampicillin. The culture was grown overnight at 37°C with shaking at 200 rpm. Five (5) mL of overnight culture were used to inoculate 600 mL of LB broth containing 100 $\mu\text{g/mL}$ ampicillin. Upon reaching an OD_{600} of between 0.5-0.8, fusion protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma, St. Louis, USA) to a final concentration of 0.3 mM. The culture was further incubated at 22°C on a rotary shaker (200 rpm) overnight and the cells harvested by centrifugation at 5,000 x g for 15 min at 4°C . The cell pellet was suspended in 35 mL of ice-cold column buffer containing 20 mM HEPES (pH 8.0), 500 mM NaCl and 1 mM EDTA. Cell breakage was performed by sonication (Fisher Model 550 Sonic Dismembrator) on ice for 15 sec followed by 30 sec cooling for 12 cycles. The sonicated crude extract was centrifuged at 12,000 x g for 30 min at 4°C . All subsequent manipulations were carried out at 4°C . The clarified supernatant was loaded at a flow rate of 0.5 mL/min onto a column containing 10 mL chitin-coated beads (New England BioLabs), previously equilibrated with column buffer, to allow binding of the fusion-protein to the beads. The column was washed with 150 column volumes of column buffer at 2 mL/min. On-column cleavage was achieved by passing 3 column volumes of column buffer containing 100 mM DTT. After 72 h, the

protein was eluted from the column with 2 bed volumes of column buffer. The eluted protein was pooled with protein collected from a second column prepared in an identical fashion and precipitated by ammonium sulfate added to 90% saturation. After centrifugation for 60 min at 17, 000 x g, the pellet was suspended in 1 mL of 25 mM Bicine buffer, pH 7.5 and 5 mM DTT to protect the enzyme from oxidative damage. Excess salts were removed using a Hitrap 5 mL desalting column (Amersham Pharmacia) equilibrated with 25 mM Bicine (pH 7.5) and 5 mM DTT. The final volume of the desalted protein was approximately 2 mL, which was aliquoted in 25 μ L volumes into sterile tubes and stored at -20°C. Enzyme activity was retained during storage under these conditions for at least 6 weeks.

2.2.4 Enzyme activity assay and effect of various conditions on enzyme activity

PcpC activity was assayed by following the disappearance of the TCHQ substrate as described by McCarty *et al.* (McCarthy et al. 1996) with some modifications. The standard assay buffer contained 25 mM Bicine (pH 8.7), 10 mM ascorbic acid, 1 mM EDTA, 25 mM DTT and 4 mM reduced glutathione (GSH). A TCHQ stock solution was prepared in assay buffer and diluted to 40 μ M for use in most experiments. Frozen protein stocks were thawed on ice and diluted 10-fold in assay buffer. Protein concentration was determined using a Coomassie Reagent assay (Pierce, Rockford, Illinois, USA) prior to each experiment. To start an enzyme assay, the assay buffer of 1 mL containing the appropriate TCHQ concentration was first incubated for 1 h at the assay temperature. The reaction was initiated by the addition of ice-cold PcpC enzyme. The volume of the enzyme added (< 0.5% of the total assay volume) did not significantly

alter the temperature of the assay mix. At various times, 100 μ L aliquots were removed from the assay mix and added to an equal volume of 1 N HCl to stop the reaction. The HCl solution contained 90 μ M *p*-nitrophenol (Sigma) that served as an internal standard for subsequent quantification of substrate and products. Samples were centrifuged at 14,000 x g for 30 sec and analyzed by HPLC using a Hypersil Green ENV column (150 mm long x 4.6 mm inner diameter) at 22°C. The substrate (TCHQ), internal standard (*p*-nitrophenol), and products were eluted by acetonitrile:water (1:1, v/v) flowing at 0.75 mL/min. TCHQ loss was estimated by comparison of peak area relative to known quantities of authentic TCHQ standard (Sigma) prepared for each set of experiments. One unit (U) of enzyme activity was defined as the amount of enzyme that degraded 1 nmol of TCHQ per min.

Enzyme activity was examined over the pH range of 6.8 to 10.0. The buffers (25 mM) used were: HEPES for pH 6.8-8.0, Bicine for pH 8.0-9.0 and glycine-NaOH for pH 9.0-10.0. The effect of temperature on enzyme activity was assayed over the range from 2 to 65°C. At each temperature, the assay was initiated by adding a small amount of ice-cold enzyme. The thermal stability of the enzyme was examined by incubating the enzyme at 2, 22, 50, and 65°C for varying times then measuring TCHQ degradation at the optimal assay temperature of 50°C. The effect of varying GSH (0-4 mM), DTT (0-100 mM) and TCHQ (0-200 μ M) concentrations on enzyme activity were examined by changing the concentration of each test compound, while keeping all other conditions unchanged.

All experiments were done in triplicate, except for the effect of pH and assay temperature experiments, which were based on duplicate experiments. Where applicable,

results were subjected to the Analysis of Variance (ANOVA) test. Statistically different results were considered to have $p \leq 0.05$.

2.2.5 Primary sequence comparison and analyses

Three comparisons were undertaken of the primary amino acid sequences of selected GSTs. Parentheses containing the Accession number of the corresponding GST follow the genus and species of the organism, except for *Arabidopsis thaliana* where the parentheses contain the GI number. The two closely related PcpC sequences of *Sphingomonas* sp. UG30 (AY057901) and *S. chlorophenolicum* (M98559) were first examined in a pair-wise alignment. Subsequently, the UG30 PcpC and other selected GSTs were subjected to two multiple sequence alignments using ClustalX (Thompson et al. 1997) and manually edited with Bioedit (version 5.0.9.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>)). The first multiple alignment included sequences of the Beta class GSTs from *E. coli* (D38497), *P. mirabilis* (U38482), and *Sphingomonas* sp. UG30. The second included Zeta class GST sequences from *Dianthus caryophyllus* (X58390), *Triticum aestivum* (AF002211), *Homo sapiens* (U86529), *Emericella nidulans* (O43123), *Caenorhabditis elegans* (Q18938), *Sphingomonas* sp. Strain RW5 (AJ224977), *Sinorhizobium meliloti* (AF109131), and *Naegleria fowleri* (U43126); Theta class GST from *Arabidopsis thaliana* (2554770); and *Sphingomonas* sp. UG30.

2.3 Results

2.3.1 Sequence Comparison and Analyses

The 744 bp ORF encoding the UG30 *pcpC* gene is preceded by a typical bacterial ribosome binding site (GAGG), similar to that seen in the *pcpC* of *Sphingobium chlorophenolicum* ATCC 39723. An AT-rich region at -10 and a consensus sequence TTGACG at -35 regions were found in the UG30 sequence. In contrast, the consensus sequence for initiation of transcription at -35 was not found in the *S. chlorophenolicum* sequence. A BLAST search of the 1.5-kb UG30 sequence upstream of *pcpC* found two high score matches. One was an expected close homology to that upstream of the *S. chlorophenolicum pcpC* gene. The second was to a part of an ORF of an unknown protein in the *Streptomyces lividans* amplifiable element AUD4. The relevance of this latter match is not apparent. A BLAST search of the 170-bp UG30 sequence downstream of the UG30 *pcpC* did not turn up any useful matches. Several short stretches (18-24 bp) of DNA that showed homology to widely disparate gene sequences were found. The results suggest that *pcpC* is transcribed as a monocistronic message. DNA sequence comparison between the two *pcpC* genes indicated 90% sequence identity (see Appendix 1).

The 248-amino acid UG30 *pcpC* gene product has a predicted MW of 28,247 Da with a pI of 4.99. The homologous PcpC from *S. chlorophenolicum* also comprised of 248 amino acids. Comparison of the primary sequences of the two PcpC proteins indicates 94% amino acid identity (see Appendix 2). Of the fourteen amino acid differences between the two, five are conservative changes (polar to polar or non-polar to non-polar), two are polar to non-polar and six are charged to polar or non-charged to

charged amino acids. The final 56 carboxy-terminal amino acids are identical between the two PcpC proteins. In this region, a putative transmembrane helix for an integral membrane protein (residues 195-210) was predicted by the method of Rao and Argos using the PC/GENE software. Whether the two PcpC proteins are membrane-bound remains to be determined. Both are members of the GST superfamily of enzymes that catalyze the detoxification of xenobiotics and reactive endogenous compounds by glutathione conjugation.

The UG30 PcpC exhibited low levels of similarity to the Zeta and Beta class sequences, with an average identity of about 15%. Low levels of sequence identity have been noted before among members of the GST superfamily of enzymes. The alignments also revealed the conservation of several amino acids necessary for activity in GSTs. These amino acids, dark-shaded in Figures 2.1a and 2.1b, include Tyr, Ser and Cys residues near the N-terminus. In the *S. chlorophenolicum* PcpC, Cys13 has been shown to be essential for activity (McCarthy et al. 1996). The equivalent Cys residue in the UG30 PcpC will likely play a similar role. A conserved Pro residue (Pro57 from the UG30 PcpC sequence) also aligns in all the Beta class sequences (Figure 2.1a) and the equivalent Pro53 in *P. mirabilis* has been shown to be important to maintain enzyme conformation (Allocati et al. 1999). An examination of the alignment of the UG30 PcpC with the *E. coli* and *P. mirabilis* GSTs showed a strict conservation of His113 and Lys114 (numbering based on the UG30 PcpC sequence, Figure 2.1a). Previous studies have found His106 and Lys107 to be essential for GSH binding in the *E. coli* and *P. mirabilis* GSTs (Allocati et al. 2000, Nishida et al. 1998). It is not known if the equivalent His and Lys residues play a similar role in the UG30 PcpC. Multiple

alignment of Zeta class GST sequences revealed a signature motif between residues 6-22 (Anandarajah et al. 2000), which contains many conserved residues residing in or near the GSH binding site (Figure 2.1b). In this region, the sequences of PcpCs from UG30 and *S. chlorophenolicum* are identical.

2.1a

Ecoli	---MKLF-YKPGACSLASHITLRESCKDFTLVSVDLMKKR	36
Pmirabilis	---MKLY-YTPGSCSLSPHIVLRETCLDFSIERIDLRTKK	36
UG30.Pcpc	MSEVSLYNYTMSICSMKTRLAMEEFCVDYNDKQVDIGFAL	40
Ecoli	LENGDDYFAVNPKGQVPAALLDDGTLLTEGVATMQYLADS	76
Pmirabilis	TESGKDFLAINPKGQVPVQLDNGDILTEGVATVQYLADL	76
UG30.Pcpc	ENFEPDYVRLNERAVVPTLVVGDR-VVINSYNIVLEAAKL	79
Ecoli	VPDRQLLAPVN---SISRYKTIEWLNYIATEL-HKGFT--	110
Pmirabilis	KPDRNLIAPPK---ALERYHQIEWLNFLASEV-HKGYS--	110
UG30.Pcpc	GNVGIPADPEVENKAALDWFEEKDQVNFQVITYGHKGVPRG	119

2.1b

Dianthus	-MSSSETQKMQLYSFSLSSCAWRVRIALHLKGLDFEYKAV	39
Triticum	----MATAKPILYGAWISSCSHRVRIALNLKGVDEYKAV	36
Homo_sapiens	----MQAGKPILYSYFRSSCSWRVRIALALKGIDYETVPI	36
Emericella	-MSTNSDLRVTLYTYFRSSCSARLRIALALRSISYTSVPI	39
Caenorhabditis	----MSNQKPVLYSYWRSSCSWRVRIALALKNVDEYKTV	36
Sphingomonas	-----MLELYHDWRSECSIKVRLCLAEKQLPWESRFV	32
Sinorhizobium	----MANETVLYDYWRSSASYRVRIALNLCGEAYRSVPV	35
Naegleria	-----MVLKLYGIYLSLTCVQRVVTTLKEKNLDFELVPV	33
Arabidopsis	-----AGIKVFGHPASIATRRVLIALHEKNLDFELVHV	33
UG30.Pcpc	-----MSEVSLYNYTMSICSMKTRLAMEEFGVDYNDKQV	34

Figure 2.1a Partial sequence alignment of two Beta class GSTs, *E. coli* (Ecoli) and *P. mirabilis* (Pmirabilis), and the UG30 PcpC (UG30.PcpC). **2.1b** Partial sequence alignment of Zeta and Theta class GSTs. Zeta class: *Dianthus caryophyllus* (Dianthus), *Triticum aestivum* (Triticum), *Homo sapiens* (Homo_sapiens), *Emericella nidulans* (Emericella), *Caenorhabditis elegans* (Caenorhabditis), *Sphingomonas* sp. Strain RW5 (Sphingomonas), *Sinorhizobium meliloti* (Sinorhizobium), and *Naegleria fowleri* (Naegleria). Theta class: *Arabidopsis thaliana* (Arabidopsis). The proposed Zeta class motif near the N-terminus is underlined. Dark shading represents alignment of identical residues while light shading represents alignment of similar residues.

Hydropathy plots, based on the method by Kyte and Doolittle (1982), indicate that much of the UG30 and *S. chlorophenolicum* PcpC sequences are identical with regard to their hydrophobicity (Figure 2.2). However, a distinctive region of separation occurs between amino acid residues 150-175 where the UG30 primary sequence is more hydrophobic compared to the *S. chlorophenolicum* sequence (Figure 2.2). This change in hydrophobicity was caused by three amino acid differences (Thr163→ Val, Val169→ Ile and Lys173→ Ala) from the *S. chlorophenolicum* to the UG30 sequence. These amino acid changes occur in the C-terminus of the protein in or near the substrate-binding site where they may affect the binding of the substrate to the UG30 PcpC.

Figure 2.2 Kyte and Doolittle hydropathy profiles of PcpC enzymes from *S. chlorophenolicum* ATCC 39723 (black) and *Sphingomonas* sp. UG30 (gray). A scan window of nine amino acids was utilized. Note the boxed region between amino acids 155 - 180 where the UG30 PcpC is more hydrophobic and may aid in better accommodation of the substrate in its active site.

2.3.2 Purification of PcpC

About 3.75 mg recombinant wild type UG30 PcpC protein were obtained from 1 litre of broth culture after affinity purification through the chitin column. Figure 2.3 shows the protein was pure with an apparent MW of 30 kDa as verified by SDS-PAGE stained with Sypro orange (Molecular Probes Inc., Eugene, OR, USA). When the purified PcpC was subjected to gel filtration through a Superdex 75 column (Amersham Pharmacia, Mississauga, ON, Canada), the functional enzyme eluted in a peak corresponding to 56 kDa. The results demonstrate the UG30 PcpC is a dimer, as are most GSTs (Sheehan et al. 2001, Vuilleumier 1997). The enzyme could be stored in 25 μ L aliquots at -20°C for up to one month without loss of activity. To maintain constant enzymatic activity, a high concentration of protein (≥ 1 mg/mL) was needed for storage. Concentrations of ≤ 0.5 mg/mL resulted in rapid loss of activity in less than a week. Boiling the enzyme for 10 min resulted in complete inactivation. UG30 PcpC activity was not affected by the presence of 1 mM EDTA in the assay buffer under optimal assay conditions.

2.3.3 Effect of experimental conditions on enzyme activity

UG30 PcpC activity was first determined at 22°C. PcpC was active over the tested pH range of 6.8 to 10, with an optimum of pH 8.7 (Figure 2.4). Enzyme activity declined rapidly to below 40% of the maximum at pH values lower than 8 or above 9.2. The pH activity profile remained the same when enzyme activity was assayed at 50°C, albeit the activity was higher.

Figure 2.3 SDS-PAGE profile of the purified UG30 PcpC. Lane 1, crude cell extract; Lane 2, proteins collected after passage through the chitin affinity column; and Lane 3, purified PcpC. Arrow A points to the PcpC-intein fusion protein at approximately 55 kDa), while arrow B points to the purified PcpC at approximately 30 kDa. Lane ST contains the MW standards.

Figure 2.4 Effect of pH on PcpC activity at 22°C. Each value is the mean of two independent experiments. Note the values at pH 8 and 9 have two data points representing the use of two different buffers at these pH values.

The activity of PcpC, assayed at the optimum pH of 8.7, increased steadily as the assay temperature increased from 2°C to a maximum at 50°C (Figure 2.5). Enzyme activity declined rapidly above 50°C, with approximately 33% loss at 55°C and nearly complete inactivation at 65°C. The activation energy of the enzyme calculated from the temperature range of 2-50°C was 0.6 kJ mol⁻¹ (Figure 2.5, inset). The thermal stability of the UG30 PcpC was examined by incubating the enzyme at 2, 22, 50 and 65°C for up to 4 h (Figure 2.6). Incubation at 2°C resulted in an initial decrease in activity from 53 U/ µg protein at time zero to 46 U/µg protein after 30 min. Activity then increased to a maximum of 67 U/ µg protein (a 26% increase from time zero) after 2 h incubation. Thereafter, activity returned to that at time zero. Incubation at 65°C resulted in 50% loss of activity after 3 min with complete inactivation after 5 min. At 22°C, PcpC activity increased initially to 68 U/µg protein (28% increase) after 60 min. With continued incubation at 22°C up to 240 min, activity returned gradually to that at time zero. When incubated at 50°C, PcpC activity decreased to 41 U/ µg protein within 1.5 min. After 15 min, PcpC activity reached a minimum of 31 U/ µg protein. Thereafter, continued incubation at 50°C for up to 240 min resulted in an increase in activity towards that at time zero.

Figure 2.5 Effect of assay temperature on PcpC activity at pH 8.7. **Inset.** Arrhenius plot of the dependence data. Each value is the mean of 2 independent measurements.

Figure 2.6 Thermal stability of PcpC. The enzyme was incubated at each of the indicated temperatures for up to 4 h. At various times, aliquots were removed and activity assayed at the optimal pH (8.7) and temperature (50°C). Each value is the mean of 3 independent measurements $\pm$ SD.

PcpC activity increased with increasing DTT concentration, reaching a maximum at approximately 30 mM DTT and activity remained high at up to 100 mM DTT (Figure 7). DTT at 25 mM has been shown to be necessary for the activity of *S. chlorophenolicum* PcpC by protecting the thiol group of Cys13 (McCarthy et al. 1996). Our experiments further showed that 25 mM DTT created conditions sufficiently reduced to permit the cycling of oxidized glutathione to reduced glutathione during TCHQ degradation. This was demonstrated in experiments with oxidized glutathione as the cofactor. In the absence of DTT, no TCHQ degradation was observed in the presence of 4 mM oxidized glutathione. In the presence of 25 mM DTT, however, TCHQ degradation occurred at similar rates with either oxidized or reduced glutathione. At 2 mM glutathione, rates of TCHQ degradation were 72.2 ± 5.6 U/ μ g protein with reduced GSH and 70.6 ± 0.3 U/ μ g protein with oxidized glutathione ($P > 0.73$). At 4 mM glutathione, rates were 69.5 ± 4.4 U/ μ g protein with reduced GSH and 73.5 ± 2.5 U/ μ g protein with oxidized glutathione ($P > 0.24$). These results suggest oxidized glutathione was reduced by DTT to permit the continued degradation of TCHQ by PcpC.

Figure 2.7 The effect of dithiothreitol (DTT) on PcpC activity. The enzyme buffer was prepared with each of the indicated DTT concentrations. The enzyme assays were carried out at the optimal pH (8.7) and temperature (50°C). Each value is the mean of 3 independent measurements $\pm$ SD.

Initial velocity studies showed that UG30 PcpC exhibited typical Michaelis-Menton kinetics with respect to both the substrate (TCHQ) and cofactor (reduced GSH). PcpC activity increased with increasing TCHQ concentration, leveling off at approximately 60 μM TCHQ (Figure 2.8a). This is significant because the *S. chlorophenolicum* wild type PcpC has been reported to experience severe substrate inhibition (Anandarajah et al. 2000). There was no evidence of inhibition of the UG30 PcpC at concentrations of TCHQ up to 300 μM . PcpC activity increased with increasing levels of reduced GSH, reaching saturation at greater than 1 mM GSH with 40 μM TCHQ present (Figure 2.8b). That indicates that at least 25-fold excess of reduced GSH to TCHQ was needed for optimal activity. The V_{max} and K_{m} values were determined from non-linear regression analyses of the initial velocity data from the plots (Figure 2.8a and b) for both TCHQ and GSH. Table 2.1 summarizes the selected functional and kinetic results for the UG30 PcpC. Similar data from studies of the *S. chlorophenolicum* PcpC are presented for comparison. The $K_{\text{cat, TCHQ}}$ (48.5 s^{-1}) and $K_{\text{cat, TCHQ}}/K_{\text{M, TCHQ}}$ ($2.39 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$) for the UG30 PcpC are about 20- and 35-fold, respectively, higher than those of the *S. chlorophenolicum* PcpC. The $K_{\text{M, GSH}}$ for the UG30 PcpC (149.2 μM) is approximately half of that reported for the *S. chlorophenolicum* PcpC (296 μM).

Figure 2.8a Effect of substrate (TCHQ) concentration on PcpC activity. Assays were carried out at pH 8.7 and 50°C in the presence of 4 mM GSH and 50 mM DTT. **2.8b** Effect of co-substrate (reduced glutathione) concentration on PcpC activity. Assays were carried out at pH 8.7 and 50°C in the presence of 40 μM TCHQ and 50 mM DTT. Kinetic parameters were determined by non-linear regression analysis of the initial velocity data, represented by the lines plotted on each graph. Each value is the mean of 3 independent experiments $\pm$ SD.

Table 2.1 Comparison of some functional and kinetic properties of PcpC from *Sphingomonas* sp. UG30 and *Sphingobium chlorophenolicum* ATCC 39723.

Selected Characteristics	Values	
	<i>Sphingomonas</i> sp. UG30	<i>S.chlorophenolicum</i> ATCC 39723
Temperature Optima (°C)	50	40 ^b
pH Optima	8.7	7.0 - 8.0 ^c
pI	4.99	5.08
Molecular weight	28, 245 Da	28, 249 Da
Specific Activity _{TCHQ} (nmoles/min/mg protein)	1.03 x 10 ⁵	122.8 ^b
K _{M, TCHQ} (μM)	20.3	35 ^d
k _{cat, TCHQ} (s ⁻¹)	48.5	2.4 ^d
k _{cat, TCHQ} /K _{M, TCHQ} (s ⁻¹ M ⁻¹)	2.39 x 10 ⁶	6.86 x 10 ⁴ ^d
K _{M, TriCHQ} (μM)	ND ^a	50 ^d
k _{cat, TriCHQ} (s ⁻¹)	ND	1.4 ^d
k _{cat, TriCHQ} /K _{M, TriCHQ} (s ⁻¹ M ⁻¹)	ND	2.8 x 10 ⁴ ^d
K _{M, GSH} (μM)	149.2	296 ^e
E _{act} (kJ mol ⁻¹)	0.6	ND

^a ND – data not available

^b value was provided in Xun et al. (1992), using wild type PcpC with TCHQ as substrate

^c value determined from Keifer et al. (2002a), using Cys13Ser mutant with TriCHQ as substrate

^d value determined from Keifer et al. (2002b), using Cys13Ser mutant with TCHQ and TriCHQ as substrates

^e value was provided in Anandarajah et al. (1999), using Cys13Ser mutant with TriCHQ as substrate

2.4 Discussion

In this study, we presented the cloning, purification, sequence analysis as well as functional and kinetic studies of the *Sphingomonas* sp. UG30 TCHQ reductive dehalogenase (PcpC) involved in PCP biodegradation. The only other bacterial PcpC enzyme that has been purified and characterized to some extent is that from *S. chlorophenolicum*. Despite their high degree of primary sequence identity (94%) and many other similarities, several remarkable and significant functional differences have been found between the two bacterial PcpC enzymes.

Genetic analyses have shown the UG30 PcpC to be very similar to its counterpart in *S. chlorophenolicum*. Both enzymes have 248 amino acids and share conserved residues that have been found to be essential for the activity of some GSTs, including the N-terminal Tyr7, Tyr 9, Ser12, Cys14, Ser15, His113, and Lys114 (based on the UG30 sequence). Typically one of these N-terminal residues is essential for GST activity and can be used to distinguish the various classes of GST (Sheehan et al. 2001). To date, only Cys13 has been found by site-directed mutagenesis to be catalytically essential for the *S. chlorophenolicum* PcpC (McCarthy et al. 1996). Currently, the *S. chlorophenolicum* PcpC has been classified as a Zeta class GST by the presence of a Zeta class N-terminus motif, carbon-carbon double bond isomerase activity, and inhibition by dichloroacetic acid (DCA) (Anandarajah et al. 2000). The UG30 PcpC has a similar N-terminus motif. This and other similarities suggest it may also belong to the Zeta class of GST superfamily; however, this placement should be regarded as tentative. The UG30 primary sequence contains other conserved residues including several N-terminal Tyr and Ser residues, as well as His113 and Lys114 that have not been examined to determine

their impact upon PcpC activity. Lys and His residues have been shown to be important for activity in the Beta class GSTs (Allocati et al. 2000, Nishida et al. 1998, Rossjohn et al. 1998). An N-terminal Tyr is conserved in the GSH binding site necessary for the activation of the GSH molecule in the Alpha, Pi, Mu, and Sigma class GSTs (Board et al. 1997), and an N-terminal Ser residue plays the same role in the Theta class GSTs, (Board et al. 1995). Furthermore, previous experience suggests the classification of bacterial GSTs is subject to change as new lines of experimental evidence or classification schemes come to light.

Previous studies have examined the reaction mechanism and kinetic parameters of the *S. chlorophenolicum* PcpC. This enzyme catalyzes the sequential removal of two chlorines from TCHQ to form first TriCHQ, followed by DCHQ (Orser et al. 1993, Xun et al. 1992). Stoichiometrically, four reduced GSH molecules are needed for the complete conversion of one molecule of TCHQ to DCHQ, resulting in the formation of 2 molecules of oxidized glutathione and 2 chloride ions (McCarthy et al. 1996, Xun et al. 1992). The formation of oxidized glutathione is a characteristic that has been observed in two other GSTs, namely LinD in *Sphingomonas paucimobilis* UT26 and LigF in *Sphingomonas paucimobilis* SYK-6 (Vuilleumier and Pagni 2002). In addition, the presence of a reducing agent, such as DTT, is needed *in vitro* to prevent oxidative damage to Cys13 and allow the proper formation of TriCHQ and DCHQ (McCarthy et al. 1996). The UG30 PcpC also catalyzes the degradation of TCHQ, requiring reduced GSH as the cofactor and DTT for activity; however, there are some striking differences between the two closely related PcpC enzymes in their functional and kinetic characteristics.

A most notable and interesting difference between the UG30 and ATCC 39723 PcpC enzymes is in their respective optimal pH and temperature values. The UG30 PcpC exhibits the highest activity at pH 8.7 and 50°C. In contrast, the optimal pH of the ATCC 39723 PcpC was originally reported to be between 6.0-7.0 and the optimal temperature was 40°C (Xun et al. 1992). A more recent study has placed the ATCC 39723 PcpC pH optimum between 7.0-8.0 (Kiefer et al. 2002). Although changes in a few amino acid residues are known to shift the T_{opt} and T_{max} of proteins by 10°C or more (Liao et al. 1986), it was not expected that the pH and temperature optima of the two closely related bacterial PcpC enzymes would differ so drastically. The reason(s) for such differences is/are not understood. One consideration may be the oxidative state of the enzyme. It has been shown that the oxidative state of a *P. mirabilis* GST affected its thermal stability (Caccuri et al. 2002); however, both PcpC enzymes were studied under similar reducing conditions in the presence of 25 mM DTT, and thus should not have been oxidized.

Comparison of the kinetic values between the UG30 and *S. chlorophenolicum* PcpC enzymes is difficult for two reasons. First, kinetic values for our UG30 PcpC were determined from TCHQ dechlorination, though most of those for the *S. chlorophenolicum* PcpC were based on TriCHQ dechlorination (Anandarajah et al. 2000). Second, it appears the assay for the *S. chlorophenolicum* PcpC was performed at a temperature below its determined optimum of 40°C, while experiments with our UG30 PcpC were performed under optimal conditions. Despite these differences in reaction conditions, an important distinction between the two enzymes is that the UG30 PcpC is not inhibited by the TCHQ substrate at concentrations (300 μM) that were about 15-fold higher than its K_M value (20.3 μM). In contrast, severe substrate (TCHQ) inhibition has

been reported for the *S. chlorophenolicum* PcpC (Anandarajah et al. 2000), although the concentration(s) of TCHQ needed to effect inhibition was not given (Anandarajah et al. 2000). This has necessitated the use of a Cys13Ser mutant enzyme capable of catalyzing the transformation of TriCHQ to GS-DCHQ for studying the kinetics of the *S. chlorophenolicum* PcpC (Anandarajah et al. 2000, Kiefer and Copley 2002, Kiefer et al. 2002). The factor(s) responsible for the different susceptibility to TCHQ inhibition between the two PcpC enzymes is presently not understood.

The $k_{cat,TCHQ}$ value of the UG30 PcpC is about 20-fold higher than that of the *S. chlorophenolicum* PcpC. Comparison of these values must take into account the two main differences in the assays of the two enzymes: (a) the use of the Cys13Ser mutant enzyme for the *S. chlorophenolicum* PcpC, and (b) the differences in assay temperature. The Cys13Ser mutant catalyzes only a partial reaction, up to the point where Cys is required, utilizing 1 equivalent of glutathione and, most importantly, is not subject to substrate inhibition (Anandarajah et al. 2000). If, for example, the substrate is TCHQ, the product formed by action of the mutant enzyme is 2,3,5-trichloro-6-S-glutathionyl-4-hydroxycyclohexa-2,4-dione (Anandarajah et al. 2000). The mutant has been used in initial velocity studies of the *S. chlorophenolicum* PcpC and is believed to exhibit kinetic parameters that do not differ much from the wild type enzyme (Anandarajah et al. 2000, Kiefer and Copley 2002, Kiefer et al. 2002). The $k_{cat,TCHQ}$ for the UG30 PcpC was determined at 50°C and that for the *S. chlorophenolicum* PcpC was measured at 25°C (Anandarajah et al. 2000, Kiefer and Copley 2002, Kiefer et al. 2002). Assaying the UG30 PcpC activity at 25°C would reduce activity by 3-fold (Fig. 4) and this means the turnover number would still be much higher than the *S. chlorophenolicum* PcpC. The

reason for the enhanced ability of the UG30 PcpC to catalyze TCHQ turnover is not known. One possibility may relate to the better accommodation of TCHQ at the substrate-binding region. Hydropathy plots have identified a slightly more hydrophobic region in or near the substrate-binding site in the UG30 PcpC, caused by three amino acid changes (Thr163→Val, Val169→Ile and Lys173→Ala) from the *S. chlorophenolicum* to the UG30 sequence. Whether or not any of these amino acid changes are important for the increased turnover number for the UG30 PcpC remains to be determined.

In summary, despite a high level of similarity in sequence and reaction, differences were observed in some of the functional and kinetic properties between the UG30 and *S. chlorophenolicum* PcpC enzymes. Studies are in progress to identify catalytically essential amino acid residues in the UG30 PcpC in order to understand its reaction mechanism and its tolerance for alkaline environments and increased assay temperatures. Of interest are the roles played by amino acid residues Tyr 7, Tyr9, Ser12, Cys14, Ser15, His113, and Lys114, all of which have been implicated as essential in many of the other GSTs. One feature that will allow for a detailed characterization of the catalytic mechanism of the UG30 wild type PcpC is the lack of substrate inhibition by TCHQ.

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CHAPTER THREE

An Examination of N-Terminal Amino Acid Residues Important for the Activity of the Tetrachlorohydroquinone Reductive Dehalogenase from *Sphingomonas* sp. UG30^c

3.1 Introduction

Bacterial reductive dehalogenases are a recently identified group of enzymes found primarily in anaerobic microorganisms. Either alone or as part of degradative catabolic pathways, the reductive dehalogenases have important roles in the degradation of recalcitrant toxic halogenated compounds often found in soil and water. Examples of their primary substrates include polychlorinated biphenyls (Krasotkina et al. 2001, Wiegel et al. 1999), chlorinated phenols (Mohn and Kennedy 1992b, van de Pas et al. 1999), and tetrachloroethene (Holliger et al. 1998, Magnuson et al. 1998, Neumann et al. 1994). A few examples of these enzymes have also been found in aerobic microorganisms. These include tetrachlorohydroquinone reductive dehalogenase (PcpC), which is the focus of this study, and dichloromethane dehalogenases (Bader and Leisinger 1994, La Roche and Leisinger 1990).

PcpC is a member of a multi-enzyme pathway able to degrade pentachlorophenol (PCP) to carbon dioxide and water (Cai and Xun 2002, Dai et al. 2003). Found in several microorganisms, the PcpC from *Sphingomonas* sp. UG30 has been the focus of our studies. *Sphingomonas* sp. UG30 was originally isolated from a PCP-contaminated soil sample for its ability to dechlorinate and mineralize PCP (Leung et al. 1997). The *pcpC* gene encoding this enzyme was cloned and expressed in *Escherichia coli* (Habash et al. 2002). The UG30 PcpC shows 95% primary sequence identity with the well-studied

^c Portions of this chapter have been submitted to The Biochemical Journal

PcpC from *Sphingobium chlorophenolicum* ATCC 39723. Despite this level of sequence similarity, we found several differences between the two highly homologous PcpC enzymes. Of note, the UG30 PcpC enzyme does not exhibit severe substrate inhibition (Habash et al. 2002). Substrate inhibition, which has been reported for the TCHQ reductive dehalogenase from *Sphingobium chlorophenolicum*, has hampered detailed kinetic studies using the wildtype enzyme (Anandarajah et al. 2000, Kiefer and Copley 2002a, Kiefer et al. 2002b).

PcpC has been classified as a member of the glutathione transferase (GST) superfamily of enzymes (Anandarajah et al. 2000). This enzyme family contains members that aid in the detoxification of xenobiotic compounds via the conjugation of glutathione. Structural and mutational studies of GSTs from different classes have revealed that the N-terminus of the enzyme contains amino acids essential for glutathione binding. Typically, the hydroxyl group of an N-terminal Tyr or Ser residue aids in stabilization of glutathione, present as a thiolate ion (Sheehan et al. 2001). In addition, the GSTs can possess an N-terminal Cys that is critical for either alignment of the glutathione in the active site (Board et al. 2003, Thom et al. 2001) or for forming a mixed disulfide bond with the glutathione thiol group (Casalone et al. 1998, Nishida et al. 1998).

The GSTs are divided into eleven separate classes with PcpC currently designated as a member of the Zeta class. This classification is based on the presence of isomerase activity, dichloroacetic acid activity and the presence of a Zeta class sequence motif (Anandarajah et al. 2000). The 3-D structures of two well-studied Zeta class enzymes, from *Arabidopsis thaliana* (Thom et al. 2001) and *Homo sapiens* (Polekhina et al. 2001), have been determined. Based on these structures, the N-terminal Ser and Cys residues

within the glutathione-binding regions in these enzymes have been proposed to play essential roles in the Zeta class GSTs. Site-directed mutagenesis studies of these two enzymes have confirmed the presence of N-terminal Ser (equivalent to Ser12 of PcpC) and Cys (equivalent to Cys14 of PcpC) that are critical for activity (Polekhina et al. 2001, Thom et al. 2001).

PcpC and the 2,5-dichlorohydroquinone reductive dehalogenase from the lindane-degrading *Sphingomonas paucimobilis* (Miyauchi et al. 1998) are two bacterial Zeta class GSTs with dehalogenating activity. Other GST classes, such as the Theta and Beta classes, also contain bacterial members. These include enzymes isolated from *Escherichia coli*, *Proteus mirabilis* (Mignogna et al. 1993), *Methylophilus* sp. strain DM11 (Bader and Leisinger 1994) and *Methylobacterium dichloromethanicum* DM4 (La Roche and Leisinger 1990). Among these, the Theta class DCM dehalogenase, also a reductive dehalogenase, possesses an essential N-terminal Ser (Ser13) (Vuilleumier and Leisinger 1996). In contrast, the Beta class GSTs do not possess a critical N-terminal Ser or Tyr (Casalone et al. 1998, Nishida et al. 1994). Instead, an N-terminal Cys (Cys10) has been shown to be vital for the formation of a mixed disulfide bond between the enzyme and the thiol group of glutathione (Casalone et al. 1998, Nishida et al. 1998). An examination of which of the Tyr, Ser or Cys residues in the N-terminus may provide information on the relationships and commonalities between various classes of bacterial GSTs.

The *Sphingomonas* sp. UG30 PcpC contains two Tyr (Tyr7 and Tyr9), two Ser (Ser12 and Ser15) and one Cys (Cys14) residue at the N-terminus. The similarity of the *Sphingomonas* sp. UG30 PcpC to other Zeta class GSTs has led to suggestions that the N-

terminal Ser12 and Cys14 are essential for activity. Indeed, in the highly homologous PcpC from *Sphingobium chlorophenolicum* ATCC 39723, N-terminal Cys13 plays a role in catalysis by aiding in the removal of glutathione from the glutathione-conjugated products (GS-TriCHQ and GS-DCHQ) (McCarthy et al. 1996, McCarthy et al. 1997). Mutation of Cys13 to Ser resulted in the accumulation of glutathione-conjugated metabolites (McCarthy et al. 1996). The other N-terminal Tyr or Ser residues have not been examined experimentally. In this study, we used site-directed mutagenesis to assess the catalytic role(s) of two Tyr (Tyr7 and Tyr9), two Ser (Ser12 and Ser15) and one Cys (Cys14) residues at the N-terminus of the *Sphingomonas* sp. UG30 PcpC. Our results indicate that Ser12 and Cys14 are essential for PcpC activity with regard to glutathione binding, while Tyr7 and Tyr9 do not appreciably affect activity. In addition, Ser15 has a structural role in the PcpC enzyme.

3.2 Materials and Methods

3.2.1 Site-directed mutagenesis of *pcpC*

The wild type *pcpC* gene encoding the UG30 tetrachlorohydroquinone reductive dehalogenase was used as the template for site-directed mutagenesis. We previously cloned this gene from genomic DNA into the expression vector pTXB1 to yield the plasmid pTXB1PcpC (Habash et al. 2002). This plasmid was maintained in *E. coli* ER2566 for expression and characterization of the gene product. Site-directed mutagenesis was carried out using the QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. The overlapping mutagenic primers utilized for each mutation are shown in Table 3.1. The mutated

plasmids from each mutagenic reaction were transformed in XL10 gold ultra-competent *E. coli* cells. The entire transformation mixture was plated onto LB agar supplemented with 100 µg/mL ampicillin. Plates were incubated overnight at 37°C. One clone was selected and the sequence of the mutated *pcpC* gene was determined in both directions using vector-specific primers. After sequence verification, the mutated pTXB1PcpC was transformed into competent *E. coli* ER2566 cells for expression of the mutant PcpC/intein tag fusion protein. Frozen stocks (in 50% v/v glycerol) of ER2566 cells carrying the mutated pTXB1PcpC plasmid were prepared and stored at –20°C for subsequent expression and purification of the mutant PcpC protein.

Primer		Sequence (5' to 3')
Tyr7Phe	Forward	GGTGGTCATATGTCTGAAGTCAGTCTCTTTAATTATACGATGTCGATCTG
	Reverse	CAGATCGACATCGTATAATTAAAGAGACTGACTTCAGACATATGACCACC
Tyr9Phe	Forward	GAAGTCAGTCTCTATAATTTCACGATGTCGATCTGTTTCG
	Reverse	CGAACAGATCGACATCGTGAAATTATAGAGACTGACTTC
Ser12Ala	Forward	CTCTATAATTATACGATGGCGATCTGTTTCGATGAAAACCCGG
	Reverse	CCGGGTTTTTCATCGAACAGATCGCCATCGTATAATTATAGAG
Cys14Ala	Forward	CTCTATAATTATACGATGTCGATCGCTTCGATGAAAACCCGGCTCGCG
	Reverse	CGCGAGCCGGGTTTTTCATCGAAGCGATCGACATCGTATAATTATAGAG
Ser15Ala	Forward	TATACGATGTCGATCTGTGCGATGAAAACCCGGCTCGCG
	Reverse	CGCGAGCCGGGTTTTTCATCGCACAGATCGACATCGTATA

Table 3.1 Overlapping mutagenic primers for site directed mutagenesis of UG30 *pcpC*.

The highlighted regions identify mutagenized codons.

3.2.2 Expression and purification of PcpC protein

Expression and isolation of the PcpC wild type and variant enzymes were carried out as described (Habash et al. 2002). Briefly, 50 μ L of *E. coli* ER2566 (pTXB1PcpC) frozen stock were inoculated into 50 mL LB broth containing 100 μ g/mL ampicillin. The culture was grown overnight at 37°C and shaken at 200 rpm. Twenty (20) mL of overnight culture were used to inoculate 2,000 mL of LB broth containing 100 μ g/mL ampicillin. Upon reaching an OD₆₀₀ of between 0.5-0.8, PcpC-intein fusion protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (Sigma, St. Louis, USA) to a final concentration of 0.3 mM. The culture was further incubated at 15°C on a rotary shaker (200 rpm) overnight and the cells harvested by centrifugation at 5,000 x g for 15 min at 4°C. The cell pellet was suspended in 30 mL of ice-cold column buffer containing 20 mM HEPES (pH 8.0), 500 mM NaCl and 1 mM EDTA. Cell breakage was performed by addition of 3 g alumina and sonication (Fisher Model 550 Sonic Dismembrator) on ice for 15 sec followed by 20 sec cooling for 12 cycles. The sonicated crude extract was clarified by centrifugation at 12,000 x g for 30 min at 4°C. All subsequent work was carried out at 4°C. Isolation of the fusion protein was achieved by passing the clarified supernatant through a column containing chitin-coated beads (New England BioLabs). The column was washed with 150 volumes of column buffer at 2 mL/min. On-column cleavage was achieved by passing 3 column volumes of column buffer containing 100 mM DTT. After 72 h, the target protein was eluted from the column with 2 bed volumes of column buffer. The eluted protein was precipitated by ammonium sulfate added to 75% saturation at 4°C. After centrifugation at 17,000 x g for 60 min, the pellet was suspended in 0.75 mL of 25 mM Bicine buffer, pH 8 and 5 mM

DTT to protect the enzyme from oxidative damage. Excess salts were removed using a Hitrap 5 mL desalting column (Amersham Pharmacia) equilibrated with 25 mM Bicine (pH 8) and 5 mM DTT. The final volume of the desalted protein was approximately 2 mL, which was aliquoted in 25 μ L volumes into sterile tubes and stored at -20°C. Enzyme activity was retained during storage under these conditions for at least 6 weeks. The proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5% polyacrylamide gel) to examine protein purity and molecular weight for the wildtype and mutants.

3.2.3 Enzyme Activity Assays: Temperature and Substrate Concentration Effects

Activities of the wildtype and mutant PcpCs were assayed by following the disappearance of the tetrachlorohydroquinone (TCHQ) substrate as described previously (Habash et al. 2002). The standard assay buffer contained 25 mM Bicine (pH 8.7), 10 mM ascorbic acid, 1 mM EDTA, 25 mM DTT, 4 mM reduced glutathione (GSH), and 100 μ M TCHQ. Briefly, frozen protein stocks were thawed on ice and diluted 6 to 10-fold in assay buffer, depending upon the concentration of the protein stock solution. Protein concentration was determined using a Coomassie Reagent assay (Pierce, Rockford, Illinois, USA) prior to each experiment. A TCHQ stock solution was prepared in assay buffer and diluted to 100 μ M for use in most experiments.

For experiments examining the effect of temperature on enzyme activity, 1.2 mL of standard assay buffer was warmed to the assay temperature of 23, 30, 37, 44, 50, or 60°C. The reaction was initiated by adding ice-cold enzyme (wildtype or mutant PcpC). The concentration of enzyme added was adjusted based on preliminary activity experiments,

ranging from 35.4 – 141.6 nM. The volume of the enzyme added (< 1% of the total assay volume) did not significantly alter the temperature of the assay mix.

The effects of pH were examined for the wildtype and Cys14Ala, Ser12Ala, and Ser15Ala mutant enzymes (i.e. the mutants exhibiting the greatest change in activity), over the pH range of 7, 7.5, 8, 8.7, 9.2 and 10 as previously described (Habash et al. 2002).

The effects of varying GSH (0.02 - 4 mM) and TCHQ (15 - 120 μ M) concentrations on enzyme activity were examined by changing the concentration of each test compound, while keeping all other conditions consistent. 1 mL of assay buffer containing the appropriate TCHQ or GSH concentration was warmed to either 37°C or 50°C before being used in the assay as described above.

The effect of dithiothreitol (DTT) was examined for the wildtype and Cys mutant. The enzyme assays were undertaken utilizing the standard assay buffer with or without 25 mM DTT added and the reaction temperature at 37°C.

For all assays, 50 μ l aliquots were removed at specific time points from the assay mix and added to an equal volume of 1 N HCl to stop the reaction. The HCl solution contained 180 μ M *p*-nitrophenol (Sigma) that served as an internal standard for subsequent quantification of substrate and products. Samples were centrifuged at 14,000 x g for 30 sec and analyzed by HPLC using a Hypersil Green ENV column (150 mm long x 4.6 mm inner diameter) at 22°C. The substrate (TCHQ), internal standard (*p*-nitrophenol), and products were eluted by acetonitrile:water (0.325:0.675, v/v) flowing at 0.75 mL/min. TCHQ loss was estimated by comparison of peak area relative to known quantities of authentic TCHQ standard (Sigma) prepared for each set of experiments. One

unit (U) of enzyme activity was defined as the amount of enzyme that degraded 1 nmol of TCHQ per min.

All experiments were made in triplicate, except where indicated. Where applicable, results were subjected to Analysis of Variance (ANOVA). Statistically significant different results were considered to have $p \leq 0.05$.

3.2.4 Mass Spectroscopic Analyses of Substrate and Reaction Products

Products derived from TCHQ degradation by wildtype and mutant PcpC enzymes were separated by HPLC and analyzed by mass spectroscopy to determine if the typical reaction products including DCHQ were observed. Samples and authentic TCHQ and DCHQ standards were initially separated through a Hypersil Green ENV column (150 mm long x 4.6 mm inner diameter) at 22°C with acetonitrile:water (0.325:0.675; v/v) at a flow rate of 0.4 mL/min. The peaks were analyzed by an Agilent 1100 series LC/MSD controlled with the Agilent Chemstation software (version Rev.A.08.04 [1008]). The MS analyzed the peaks in Scan mode with a negative polarity in API-ES ionization mode. The optimal conditions used during analysis were: drying gas, 10 L/min; nebulizer pressure, 45 psig; and temperature, 300°C. The fragmentor voltage was set to 60 V and the scan range of ions was set to 50-650 m/z .

3.2.5 Circular Dichroism (CD) Spectroscopy

For secondary and tertiary structure prediction, protein samples were prepared by performing a buffer exchange into 5 mM Tris containing 5 mM DTT at pH 8.7. For secondary structure analyses, a protein concentration ranging from 0.05 to 0.15 mg/mL

was utilized. For tertiary structure analysis, a protein concentration ranging from 0.5 - 1.5 mg/mL was utilized. Appropriate buffer blanks were also prepared. Protein samples and blanks were centrifuged and degassed prior to analysis. For secondary structure analysis in the far UV (250-185 nm) region, samples were placed in a quartz cuvette with a 0.1 cm path length. For tertiary structure analysis in the near UV (320-250 nm), samples were placed in a quartz cuvette with a 1 cm path length. Each sample was scanned four times at room temperature under continuous nitrogen flush with a Jasco 600 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The circular dichroism data were converted to molar ellipticity (θ).

3.3 Results

In this study, we characterized structural and functional aspects of the TCHQ reductive dehalogenase (PcpC) from *Sphingomonas* sp. UG30. Site-directed mutagenesis was used to alter five of the N-terminal residues hypothesized to play a role in cofactor binding in the UG30 PcpC. Conservative substitutions were used in each instance to minimize any potential structural perturbations. For example, the two Tyr residues were converted to Phe, while Ser and Cys residues were converted to Ala. Sequencing analyses using vector-specific primers upstream and downstream of the *pcpC* gene confirmed the correct mutation had been incorporated and that no other mutation has been introduced before expression and characterization of each variant. Each mutant protein was expressed in *E. coli* and purified as previously described (Habash et al. 2002). Typical yields for the wildtype and mutants were approximately 2 mg purified protein/L of culture. SDS-PAGE analyses indicated all the proteins were purified to

homogeneity with the expected molecular weight of approximate 28 kDa (see Appendix 3).

The purified recombinant wildtype and mutant PcpC enzymes were subjected to circular dichroism (CD) studies to determine if any of the mutants exhibited major secondary or tertiary structural changes. Figure 3.1a presents the far UV CD spectra for the wildtype and mutant enzymes. Slight variations from the wildtype spectrum were observed for each of the mutants. These variations do not, however, suggest major secondary structural changes in any of the PcpC mutants, as the extent of the variations are similar to site-directed mutants of a *Proteus mirabilis* GST in another study (Caccuri et al. 2002). In addition, the CD results verified that the PcpC variants were not misfolded based on their similarity to the wildtype enzyme. It is noted that for the Ser12Ala mutant, the decrease in the peak at approximately 190 nm and increases of the minima at 208 and 222 nm may be indicative of a loss of α -helical content and an increase in β -sheet content of the enzyme. With regard to the near UV CD spectra for mutant Ser15Ala, there appears to be an increase in the molar ellipticity in the 260-280 nm region, relative to the wildtype spectrum (Figure 3.1b). This is indicative of a change in tertiary structure that likely arises from the loss of hydrogen bonding from the hydroxyl group of Ser. Otherwise, the wildtype and mutant enzymes possess similar tertiary structures (Figure 3.1b).

Figure 3.1a Examination of PcpC wildtype and N-terminal mutant secondary structure by far UV circular dichroism. **3.1b** Examination of PcpC wildtype and N-terminal mutant tertiary structure by near UV circular dichroism.

Our preliminary examinations of the activities of all mutants were determined under the optimal enzyme assay conditions of the wildtype (i.e. at pH 8.7 and 50°C assay temperature, as determined previously in Habash et al. (2002)). The Ser12Ala, Ser15Ala and Cys14Ala mutants displayed drastically decreased activity relative to the wildtype, while activities of Tyr7Phe and Tyr9Phe variants were not different from the wildtype. Thus, we examined the temperature- and pH-activity profiles for the Ser12Ala, Ser15Ala and Cys14Ala mutants to determine how they differed from the wildtype UG30 PcpC. The temperature-activity profiles showed the temperature optima for the Ser12Ala, Ser15Ala and Cys14Ala mutants shifted to lower values relative to the wildtype (Figure 3.2). The optimum for the wildtype is approximately 50°C (Habash et al. 2002), while the optima approached 40°C for Ser15Ala and 44°C for Ser12Ala and Cys14Ala. In addition, the apparent $T_{\max}$ for Ser12Ala, Ser15Ala and Cys14Ala mutants appears to have shifted to a lower temperature when compared to the wildtype enzyme (Figure 3.2). The loss of suspected hydrogen bonding by mutating Ser12, Cys14, and Ser15 to Ala would suggest they play a role in the thermo-tolerance of the enzyme, as observed by the decrease in T_{opt} and the apparent decrease in $T_{\max}$. Studies of ribonuclease T1 have indicated the important role hydrogen bonding plays in the thermostability of enzymes (Shirley et al. 1992). In addition, changes in temperature optima due to single amino acid changes have been noted previously for the kanamycin nucleotidyltransferase from *Bacillus stearothermophilus* (Liao et al. 1986). The effect of pH was also compared for the wildtype and serine and cysteine mutants at 37°C (Figure 3.3). There was a small shift (0.3 pH units) in the pH optimum for the mutants (9) compared to the wildtype enzyme (8.7).

Figure 3.2 Effect of temperature on PcpC wildtype and serine and cysteine mutant activity. Comparison of wildtype and mutant temperature curves (wildtype data presented previously in (Habash et al. 2002)); **Inset** PcpC serine and cysteine mutants. Reaction conditions: 25 mM Bicine (pH 8.7), 100 μ M TCHQ, 4 mM GSH, 25 mM DTT, 12 mM ascorbic acid, 1 mM EDTA, and 1 μ g/mL protein. Results for the mutants are averages and standard deviations from at least three independent determinations. Legend: Wildtype (∇); Ser12Ala ($\blacktriangledown$); Cys14Ala ($\bullet$); and Ser15Ala ($\circ$).

Figure 3.3 Effect of pH on PcpC wildtype and serine and cysteine mutant activity. Comparison of wildtype and mutant pH curves obtained at 37°C; **Inset** PcpC serine and cysteine mutants. Reaction buffers: 25 mM HEPES (pH 7); 25 mM Bicine (for pH 7.5 - 8.7); and 25 mM Glycine/NaOH (pH (9.2 – 10). Reaction parameters held constant over pH range: 100 μM TCHQ, 4 mM GSH, 25 mM DTT, 12 mM ascorbic acid, 1 mM EDTA, and 1 μg/mL protein. Results are averages from two independent determinations. Legend: Wildtype (▽); Ser12Ala (▼); Cys14Ala (●); and Ser15Ala (○).

Kinetic studies of TCHQ degradation as a function of varying TCHQ concentrations while keeping GSH concentration constant were examined at 37°C and 50°C. A striking observation of the kinetic data is the substrate inhibition of the Tyr7Phe, Ser12Ala, and Ser15Ala mutants at 37°C (Figure 3.4). In contrast, the Tyr7Phe, Ser12Ala, and Ser15Ala mutants were not inhibited by increasing concentrations of TCHQ at 50°C (Figure 3.5). The wildtype and Tyr9Phe and Cys14Ala mutants were not inhibited by TCHQ at either 37°C or 50°C. It is not clear why these mutations result in the inhibition of PcpC by increasing concentrations of TCHQ only at 37°C. Studies of the wildtype ATCC PcpC have indicated the enzyme experiences severe substrate inhibition, which has been linked with the N-terminal Cys present in the glutathione binding site. Although a definitive explanation for the substrate inhibition has not been presented, mutation of the ATCC PcpC Cys to Ser alleviated the substrate inhibition. It should be noted, however, that the reaction was affected, allowing only a single chloride ion to be removed from the substrate, rather than the two chloride ions typical of the wildtype mechanism.

Figure 3.4 Effect of TCHQ concentration on PcpC wildtype and site directed mutant activity at 37°C. Reaction conditions: 25 mM Bicine (pH 8.7), 4 mM GSH, 25 mM DTT, 12 mM ascorbic acid, 1 mM EDTA. Results for the mutants are averages and standard deviations from at least three independent determinations. Legend: Wildtype (●); Tyr7Phe (□); Tyr9Phe (▼); Ser12Ala (▽); Cys14Ala (■); and Ser15Ala (○).

Figure 3.5 Effect of TCHQ concentration on PcpC wildtype and site directed mutant activity at 50°C. Reaction conditions: 25 mM Bicine (pH 8.7), 4 mM GSH, 25 mM DTT, 12 mM ascorbic acid, 1 mM EDTA. Results for the mutants are averages and standard deviations from at least three independent determinations. Inset. Expanded view of the PcpC Ser and Cys mutants. Legend: Wildtype (●); Tyr7Phe (○); Tyr9Phe (▼); Ser12Ala (▽); Cys14Ala (■); and Ser15Ala (□).

With respect to the UG30 PcpC kinetics at 37°C, there were no significant changes in the K_m values for TCHQ for any of the mutants (Table 3.2). The Ser12Ala mutant had the largest change in K_m with an approximately 2.5-fold lower value than the wildtype. The Cys14Ala mutant exhibited a 9.6-fold decrease in k_{cat}/K_m relative to the wildtype, due in part to its lowered k_{cat} value (6.9-fold decrease relative to the wildtype). At 50°C, the k_{cat} values of the Tyr7Phe and Tyr9Phe mutants did not differ significantly from the wildtype. The Ser12Ala, Ser15Ala and Cys14Ala mutants showed significantly decreased (ranging from 17.5- to 35-fold) k_{cat} value, while the K_m values did not change as much (ranging from 0.67- to 3.7-fold) relative to the wildtype. Thus, for these mutants, the main apparent effect of the mutations was a reduction in the reaction rate, rather than a change in the affinity for TCHQ. This led to k_{cat}/K_m values of these mutants ranging from 23.2- to 80.3-fold lower than the wildtype value.

Kinetics studies of TCHQ degradation as a function of varying GSH concentrations while keeping TCHQ concentrations constant were performed at 37 and 50°C (Table 3.2). At 37°C, neither of the two Tyr mutants showed any significant kinetic changes relative to the wildtype. The k_{cat} of the Ser12Ala mutant was 8.2-fold lower, while the K_m was 1.28-fold higher than the wildtype. Taken together, these results further suggest that Ser12 may play a role in the reaction rate and not cofactor binding. In contrast, the Cys14Ala and Ser15Ala mutants showed a similar effect in that their k_{cat} values decreased slightly, by 3- and 2-fold, respectively, while the K_m increased considerably, by 25.4- and 6.4-fold, respectively, as compared to the wildtype. This indicates a large decrease in the affinity of these mutants for the cofactor GSH. These results are not surprising as Ser12 and Cys14 are located in the glutathione-binding region and their

mutations are expected to affect cofactor binding, as anticipated from a comparison with the crystal structures of the human and *A. thaliana* Zeta GSTs (Polekhina et al. 2001, Thom et al. 2001).

At 50°C, a common feature seen in all of the mutants is a more than 5-fold increase in their K_{mGSH} values, correlating to a decrease in their affinities for GSH (Table 3.2). This underscored the importance of the N-terminal region in GSH binding. Site-specific mutations in this region can result in less stable interactions with the cofactor and this effect was more pronounced at the higher temperature. The K_m for GSH for the Tyr7Phe and Tyr9Phe variants increased by 7.2- and 5.7-fold, respectively, but the k_{cat} values remained unchanged, as compared to the wildtype. This differs from the trends seen at 37°C, where K_m values for the two Tyr mutants were similar to the wildtype. The results suggest that the two Tyr residues may play a supporting role in GSH binding, which only became apparent at higher temperatures where the mutants are less stable. The Ser12Ala, Ser15Ala and Cys14Ala variants all exhibited much reduced (15- to 30-fold) k_{cat} values than the wildtype. Similar to the trends at 37°C, the K_m for GSH for the Cys14Ala and Ser15Ala variants were higher than the wildtype. With regard to the Ser12Ala mutant, the K_m for GSH increased 10-fold from 37°C to 50°C. The large increase in K_m for Ser12Ala suggests this mutant was structurally less stable at higher temperatures.

Table 3.2 Kinetic parameters of TCHQ degradation by PcpC wildtype and N-terminal mutants at 37 and 50°C. Results are averages and standard errors from at least three independent determinations.

Protein	Kinetic Results at 37°C				Kinetic Results at 50°C			
	k_{cat} (s ⁻¹)	K_m , TCHQ (μ M)	K_m , GSH (mM)	k_{cat}/K_m , TCHQ ($\times 10^5$ s ⁻¹ M ⁻¹)	k_{cat} (s ⁻¹)	K_m , TCHQ (μ M)	K_m , GSH (mM)	k_{cat}/K_m , TCHQ ($\times 10^5$ s ⁻¹ M ⁻¹)
Wildtype ^a	9.56 $\pm$ 0.3	11.4 $\pm$ 0.6	81 $\pm$ 11	8.39	42 $\pm$ 0.9	17.4 $\pm$ 0.7	149 $\pm$ 22	24.1
Tyr7Phe	10.1 $\pm$ 10.3 ^b	17.2 $\pm$ 33.4 ^b	71.2 $\pm$ 12.7	5.90	42.9 $\pm$ 10.4	51.4 $\pm$ 28.1	1,066 $\pm$ 255	8.35
Tyr9Phe	10.9 $\pm$ 5.3	6.1 $\pm$ 11.1	52 $\pm$ 16.6	17.85	31.9 $\pm$ 3.6	22.1 $\pm$ 9.4	847 $\pm$ 303	14.46
Ser12Ala	6.3 $\pm$ 4.2 ^b	31.1 $\pm$ 30.5 ^b	103.3 $\pm$ 46.7	2.02	1.8 $\pm$ 0.1	50 $\pm$ 6.9	1,090 $\pm$ 177	0.36
Cys14Ala	1.4 $\pm$ 0.1	16 $\pm$ 3.8	2,060 $\pm$ 896	0.87	2.1 $\pm$ 0.3	51.3 $\pm$ 18	838 $\pm$ 397	0.41
Ser15Ala	5.17 ^c	10 ^c	527 $\pm$ 49.6	5.2	1.9 $\pm$ 0.1	39 $\pm$ 6.9	895 $\pm$ 527	1.04

^a kinetic results for 50°C were obtained from (Habash et al. 2002).

^b – k_{cat} and K_m values for Tyr7Phe and Ser12Ala at 37°C are estimates due to substrate inhibition.

^c – k_{cat} and K_m values for Ser15Ala at 37°C are estimates due to substrate inhibition. To note, Ser15Ala appeared to be severely inhibited making non-linear regression analyses unsatisfactory. Thus, V_{max} for Ser15Ala was taken to be the peak value from plot in Figure 4 and the K_m was estimated based upon the peak.

The role of an N-terminal Cys in the GSTs has been shown to be important for enzyme activity in a variety of capacities including the oxidative state of GSTs in Beta and Pi class GSTs (Caccuri et al. 2002, Caccuri et al. 2002, Lo Bello et al. 1998); disulfide bond formation in the Beta class GSTs (Nishida et al. 1998, Rossjohn et al. 1998), GSH orientation in the Zeta class GSTs (Board et al. 2003, Polekhina et al. 2001, Thom et al. 2001); and for removal of the conjugated GSH from GS-TriCHQ and GS-DCHQ in the ATCC PcpC (McCarthy et al. 1996). Our previous study (Habash et al. 2002) reported that the presence of dithiothreitol (DTT) was essential for the activity of the wildtype UG30 PcpC enzyme, suggesting the involvement of a Cys residue in catalysis. In the current study, it has been shown that Cys14 in the UG30 PcpC is involved in the binding of GSH in the active site. Additional experiments specifically examined the role of Cys14 with respect to the oxidative state of the UG30 PcpC (in the presence and absence of DTT) and its impact on the metabolic products formed during TCHQ degradation via their analyses by LC/MS.

In this study we found the impact of DTT on the activity of the UG30 PcpC wildtype was reflected in the reaction rate rather than the distribution of metabolic products. In the absence or presence of DTT no glutathione-conjugated metabolites were found by LC/MS analysis of the reaction products. With DTT the wildtype UG30 PcpC converted 100 μ M TCHQ to TriCHQ and ultimately to DCHQ within 40 minutes. In the absence of DTT the wildtype enzyme reaction rate was inhibited and only TriCHQ accumulation was observed in the 4-hour experiment. With regard to the Cys14Ala UG30 PcpC mutant the presence or absence of DTT had no effect on either the rate of the reaction or the distribution of the metabolites formed. Similar to the wildtype enzyme,

the predominant metabolites observed were TriCHQ and DCHQ, as verified by similar retention times to authentic standards and identified by LC/MS (Figure 3.6). This differs from the highly homologous PcpC from ATCC 39723 in two important respects. First, in the absence of DTT the wildtype ATCC PcpC predominately produces glutathione-conjugated metabolites (GS- TriCHQ and GS-DCHQ) with no apparent change in reaction rate (McCarthy et al. 1996). Second, the ATCC PcpC Cys13Ser mutant activities have been shown to only produce GS-TriCHQ and GS-DCHQ, but no DCHQ as measured by HPLC and mass spectroscopy of products formed in during the catalysis of TCHQ with the Cys13Ser mutant enzyme (McCarthy et al. 1996). Thus, the Cys residue in each of these two PcpC enzymes may play different roles during catalysis of TCHQ.

Figure 3.6 Representative plots of the LC/MS data obtained for the wildtype and Cys14Ala PcpC enzymes in the presence and absence of DTT. The compounds were eluted on a Hypersil Green ENV column (150 mm long x 4.6 mm inner diameter) at 22°C in acetonitrile:water (0.325:0.675, v/v) flowing at 0.75 mL/min and detected at 309 nm. Note the retention times of the standards: 25 PPM TCHQ 12.5 mins; 25 PPM TriCHQ 8.5 mins; 25 PPM DCHQ 6 mins; and internal standard (*p*-nitrophenol) 8 mins. The peaks located between 2 and 4 minutes are water soluble components of the enzyme reactions including GSH, Bicine, EDTA, and ascorbic acid. No glutathione conjugated products were detected by mass spectroscopy.

3.4 Discussion

The Zeta class of GST enzymes comprises of members derived from diverse organisms ranging from bacteria, fungi, plants and animals (Board et al. 1997). The Zeta class GSTs from human and *Arabidopsis thaliana* have been studied in some detail and their 3-D structures have been resolved (Polekhina et al. 2001, Thom et al. 2001). For both enzymes, two N-terminal amino acids (Ser and Cys) have been identified as being important for enzyme activity. An inspection and comparison of the N-terminal sequence of the PcpC from *Sphingomonas* sp. UG30 with several other GSTs have shown the conservation of several potentially important amino acids in the glutathione binding region, including Tyr7, Tyr9, Ser12, Cys14 and Ser15 (Habash et al. 2002). All of those residues were targeted for site-directed mutagenesis in this study. Our results showed that Ser12 and Cys14 may be directly involved in GSH binding and are essential for PcpC activity. In addition, we have found Ser15, presumably also in the glutathione-binding region, has some as yet undefined role in the function of the UG30 PcpC. Tyr7 and Tyr9 did not participate directly in PcpC activity; however, they may play a supporting role in GSH binding which became apparent at higher temperatures (50°C) where the mutant enzymes (Tyr7Phe and Tyr9Phe) exhibited considerably lower affinity for GSH.

N-terminal active site Ser residues have been implicated in stabilizing the GSH thiolate in both Zeta and Theta class GSTs (Sheehan et al. 2001). For example, Ser14 located at the N-terminus of the α 1-helix in the human Zeta class GST forms a H-bond with the main chain carbonyl of Tyr11 and with Ser17 via its main chain and its side chain (Figure 3.7) (Board et al. 2003, Polekhina et al. 2001).

Figure 3.7 Ribbon diagram of Domain I of the human Zeta glutathione transferase/maleylacetoacetate isomerase (EC 5.2.1.2) indicating the hydrogen bonding (dashed lines) between Ser14, Tyr11, and Ser17. The N-terminus of the enzyme is also identified.

To fulfill its catalytic role, Ser14 is thought to undergo a conformational shift for it to be able to face the glutathione-binding region and participate in stabilizing the thiolate ion (Polekhina et al. 2001). A Ser14Ala mutant of the human zeta GST exhibited little activity against several substrates and this was attributed to altered conformation due to a lack of H-bonding with Tyr11 and Ser17 (Board et al. 2003). In addition those authors suggested that this mutation would lead to a more thermally labile enzyme (Board et al. 2003). Our results for the equivalent Ser12Ala variant of the UG30 PcpC indicate that this may be the case, as this mutant exhibited a lower temperature optimum relative to the wildtype (Figure 3.3). In addition, we have noted a shift in its secondary structure (Figure 3.1). The cause of the shift in secondary structure may reflect the inability of the

Ser12Ala mutant to H-bond with Tyr9 and Ser15 in PcpC, a scenario speculated for the human Zeta GST (Board et al. 2003). The Ser12Ala PcpC variant apparently can maintain a conformation capable of recognizing GSH, as seen in the K_m for GSH being very close to the wildtype at 37°C. Note, however, that the large decrease in k_{cat} of Ser12Ala relative to the wildtype suggests the –OH group of Ser12 may play a direct role in the activity of PcpC, possibly by stabilizing the GSH thiolate ion in the GSH binding region.

Based upon the 3-D structure of the human Zeta GST (Polekhina et al. 2001), it has been speculated that the N-terminal Cys residue does not play a direct role in catalysis due to the fact that it is buried within the GSH binding site. This would place the Cys too distant to form a disulfide bond with the GSH molecule in the active site. In the PcpC from *Spingobium chlorophenolicum* ATCC 39723, Cys13 has been shown to be essential for the production of TriCHQ and DCHQ (McCarthy et al. 1996). During the reaction catalyzed by the ATCC 39723 PcpC, chlorines are removed initially from TCHQ and subsequently from TriCHQ by glutathione conjugation (Kiefer and Copley 2002a, Kiefer et al. 2002b, McCarthy et al. 1996). The glutathione is removed from the glutathione-conjugated metabolites by forming a mixed disulfide bond with the N-terminal Cys13 (McCarthy et al. 1997). Reaction of the glutathione/PcpC adduct with free reduced glutathione generates glutathione disulfide and the free enzyme (McCarthy et al. 1997). This mechanism was supported by site-directed mutagenesis study whereby a Cys13Ser mutant is only capable of catalyzing the removal of a single chlorine from TCHQ (McCarthy et al. 1996). The absence of the thiol group in the Cys13Ser variant does not allow the glutathione to be removed from the glutathione-conjugated metabolite.

In comparison to the ATCC Cys13Ser mutant, activity of the UG30 PcpC was greatly affected by the mutation of Cys14. In the Cys14Ala variant, both the k_{cat} and affinity for GSH were greatly reduced. This variant, however, retained the ability to carry out stepwise dechlorination of TCHQ to TriCHQ and then to DCHQ, as confirmed in LC/MS studies where the reaction products for the Cys14Ala variant were TriCHQ and DCHQ, rather than glutathione-conjugated metabolites. Thus, Cys14 in the UG30 PcpC appears to be necessary for the proper orientation or stabilization of the GSH molecule in the active site, and not directly involved in the reaction mechanism, which correlates with what has been observed for other Zeta class GSTs.

The susceptibility of the UG30 PcpC wildtype and Cys14Ala mutant enzymes to oxidation was examined and shown to differ from what has been observed previously for the *S. chlorophenolicum* ATCC 39723 PcpC. In the absence of DTT, oxidative damage to the ATCC 39723 PcpC wildtype enzyme results in the formation of glutathione-conjugated products with no apparent change in reaction rate (McCarthy et al. 1996). In contrast, in this and a previous study (Habash et al. 2002) we have shown the activity of the UG30 PcpC wildtype was essentially non-existent in the absence of DTT. Inactivation of the enzyme in the absence of DTT could be the result of disulfide bond formation within the glutathione binding site. Modeling of a Pi class GST has indicated that the inactive oxidized form of the enzyme results in the formation of a disulfide bond between Cys47 and Cys101 within the glutathione binding site (Lo Bello et al. 1998). Our results with DTT and site-directed mutagenesis of Cys14 suggest a similar event may be occurring in the UG30 PcpC. The oxidization of the UG30 PcpC is likely because of a conformational shift that brings Cys14 and Cys158 (the only two Cys residues present in

the enzyme) together to form a disulfide bond that effectively alters the glutathione binding site to prevent catalysis.

Our results indicated that once the Cys14 was mutated to Ala, replacing the Cys sulfhydryl group with a hydrogen atom, DTT was no longer required to maintain enzyme function. This suggests that Cys14 does play a role in the inactivation of the UG30 PcpC via oxidation, whereby Cys14 and Cys158 would be unable to form a disulfide bond regardless of the presence or absence of DTT. Interestingly, the mutation of Cys158 could theoretically result in an enzyme still capable of dehalogenating its substrates without the need for a reducing agent or reduced environment. Mutation of the equivalent Cys156 in the ATCC 39723 PcpC did not appear to adversely affect activity (McCarthy et al. 1996). Such a mutant enzyme that allows dehalogenation to occur under conditions that are relatively easy to maintain would have good potential for environmental applications.

In the UG30 PcpC, the Ser15Ala mutant exhibited marked decrease in k_{cat} and affinity for GSH. This suggests that this amino acid may also have some role in GSH binding, although the precise nature of this role is not known. Resolving the 3-D structure for PcpC should help in deciphering how Ser15 participates in the activity of the UG30 PcpC. One clue resides in the equivalent Ser17 in the human Zeta class GST. Ser17 is believed to aid in maintaining the structure of the human Zeta class GST through interaction of its main and side chain atoms with Ser14 (Board et al. 2003).

Among other bacterial GSTs, dichloromethane (DCM) dehalogenase from *Methylophilus* sp. strain DM11 (a member of the Theta class of GST) shows several similarities to PcpC. Functionally, both bacterial enzymes catalyze reductive

dehalogenation reactions through glutathione conjugation. Our pairwise alignment of DM11 DCM dehalogenase and UG30 PcpC indicated an identity of 16%. Despite this low level of identity, we observed the alignment of a Ser and a Cys located at the N-terminus of both sequences. The Ser residue corresponded with Ser12 in the UG30 PcpC and Ser13 of the DM11 DCM dehalogenase. Site-directed mutagenesis studies with the DM11 DCM dehalogenase revealed that Ser13 was critical for activity, by aiding to stabilize the glutathione thiolate in the glutathione binding site (Vuilleumier and Leisinger 1996). That suggests a similar role for the UG30 PcpC Ser12. Interestingly, DM11 DCM dehalogenase Cys16 aligns with the Cys14 of the UG30 PcpC. Thus, the N-terminal Cys residue also has the potential to be important for activity in the DM11 DCM dehalogenase, as we have shown for Cys14 of the UG30 PcpC. To date, however, the role of Cys16 in the DM11 enzyme has not been elucidated.

It has become clear through our studies on the UG30 PcpC (this study and experiments presented in Chapter 2) and its comparison to the ATCC 39723 PcpC that there are some major differences in the functional and kinetic properties of these highly homologous enzymes. Both enzymes have in common the requirement of a reduced environment and glutathione for the dehalogenation of TCHQ to DCHQ via TriCHQ. Despite this mutual base these enzymes differ in their pH and temperature optima. As well, the UG30 PcpC does not experience the severe substrate inhibition described for the ATCC 39723 PcpC. Lastly, our study has raised questions about the role(s) played by Ser12 and Cys14 in each of the PcpC enzymes.

3.5 References

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CHAPTER FOUR

The Role of Histidine and Lysine Residues in Tetrachlorohydroquinone Reductive Dehalogenase from *Sphingomonas* sp. UG30^d

4.1 Introduction

Tetrachlorohydroquinone (TCHQ) reductive dehalogenase (PcpC) is a member of the microbial dehalogenases that have central roles in the degradation of recalcitrant toxic halogenated compounds found in soil and water. The PcpC we have been studying comes from *Sphingomonas* sp. UG30, a bacterial strain originally isolated from a soil sample obtained from a pentachlorophenol-contaminated site in Ontario, Canada (Leung et al. 1997). The UG30 PcpC is part of a proposed multiple enzyme pathway able to mineralize high levels of pentachlorophenol to carbon dioxide and water. PcpC catalyzes the stepwise dechlorination of tetrachlorohydroquinone (TCHQ) to trichlorohydroquinone (TriCHQ) and then to dichlorohydroquinone (DCHQ). The dechlorination reaction requires reduced glutathione (see Chapter 2) and, thus, PcpC has been classified as a member of the glutathione transferase (GST) superfamily (Xun 1992a).

The GSTs are divided into eleven classes. An examination of the reaction mechanism from various GST classes has indicated that His and Lys residues play several key roles. For example, computer modeling of the dichloromethane (DCM) dehalogenase, a reductive dehalogenase in the Theta class GST, from *Methylophilus* sp. strain DM11 indicated that H116 in conjunction with Trp117 could aid in the positioning of DCM in the active site (Marsh and Ferguson 1997). His116 subsequently aids in the

^d A condensed version of this chapter has been submitted to FEBS Letters.

dechlorination step by donating a proton, thus releasing Cl^- as HCl (Marsh and Ferguson 1997). His106 and Lys107 of the Beta class GST from *Proteus mirabilis* have been shown to aid in the orientation of the GSH in the binding site (Allocati et al. 2000). Also, a His residue (His107) has been shown to stabilize the negatively charged glutathione thiolate in a human Mu class GST (Caccuri et al. 1999) and the same function has been proposed for His106 of the Beta class GST from *Escherichia coli* (Nishida et al. 1998). Sequence comparisons of PcpC with other bacterial GSTs indicated the conservation of His113 and Lys114 residues found to be important for activity in *P. mirabilis* and *E. coli* (see Chapter 2). Thus, there is a potential for His and/or Lys residues to play a role in the activity of PcpC.

In the present study, we used chemical modification and site-directed mutagenesis to examine the role of His113 and Lys114. Our preliminary chemical modification studies with diethyl pyrocarbonate (DEPC) showed PcpC activity decreased substantially in the presence of DEPC suggesting the role of histidine residues in the functioning of PcpC. Thus, we modified, via site directed mutagenesis, the four histidines present in PcpC as well as Lys114, corresponding to Lys107 in *P. mirabilis*, to determine their impact on the activity of PcpC. Our results show that mutants Lys114Ala and His154Ala had about an 8.5-fold increase in K_m over the wildtype enzyme, indicating these amino acids may play roles in the orientation of glutathione in the active site.

4.2 Materials and Methods

4.2.1 Site directed mutagenesis of *pcpC*

The wild type *pcpC* gene encoding the UG30 TCHQ reductive dehalogenase was used as the template for site-directed mutagenesis. Previously, we have cloned this gene from genomic DNA into the expression vector pTXB1 to yield the plasmid pTXB1PcpC (Habash et al. 2002). This plasmid was maintained in *E. coli* ER2566 for expression and characterization of the gene product. Site-directed mutagenesis was carried out using the QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. Five site-directed mutants were constructed using the overlapping mutagenic primers presented in Table 4.1. The mutated plasmids from each mutagenic reaction were transformed in XL10 gold ultra-competent cells. The entire transformation mixture was plated onto LB agar supplemented with 100 µg/mL ampicillin. Plates were incubated overnight at 37°C. One clone was randomly selected and the sequence of the mutated *pcpC* gene was determined in both directions using vector-specific primers. After sequence verification, the mutated pTXB1PcpC was transformed into competent *E. coli* ER2566 cells for expression of the mutant PcpC/intein tag fusion protein. Frozen stocks (in 50% v/v glycerol) of ER2566 cells carrying the mutated pTXB1PcpC plasmid were prepared and stored at -20°C for subsequent expression and purification of the mutant PcpC protein.

Primer	Direction	Sequence (5' to 3')
His113Ala	F	GGTCATCACCTATGGCGCCAAGGGCGTGCCGCGC
	R	GCGCGGCACGCCCTTGGCGCCATAGGTGATGACC
Lys114Ala	F	GGTCATCACCTATGGCCACGCGGGCGTGCCGCGCGGGGACG
	R	CGTCCCCGCGCGGCACGCCCGCGTGGCCATAGGTGATGACC
His148Ala	F	CCATTTACCAAGCCGCGGCCGATCGCATCGTGGAGC
	R	GCTCCACGATGCGATCGGCCGCGGCTTGGTAAATGG
His154Ala	F	CGATCGCATCGTGGAGGCTGGCAATTGCGCCTATGACG
	R	CGTCATAGGCGCAATTGCCAGCCTCCACGATGCGATCG
His181Ala	F	GACGAACTGGACGCGGCTCTGGCGGACAAGCCG
	R	CGGCTTGTCCGCCAGAGCCGCGTCCAGTTCGTC

Table 4.1 Overlapping mutagenic primers for site directed mutagenesis of UG30 *pcpC*.

The highlighted regions identify mutagenized codons.

4.2.2 Expression and purification of PcpC protein

Protein expression of the PcpC-Chitin binding domain fusion protein was as described previously (Habash et al. 2002). Fifty (50) μ L of *E. coli* ER2566 (pTXB1PcpC) frozen stock were inoculated into 50 mL LB broth containing 100 μ g/mL ampicillin. The culture was grown overnight at 37°C and shaken at 200 rpm. Twenty (20) millilitres of overnight culture were used to inoculate 2, 000 mL of LB broth containing 100 μ g/mL ampicillin. Upon reaching an OD₆₀₀ of between 0.5-0.8, fusion protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (Sigma, St. Louis, USA) to a final concentration of 0.3 mM. The culture was further incubated at 15°C on a rotary shaker (200 rpm) overnight and the cells harvested by centrifugation at 5, 000 x g for 15 min at 4°C. The cell pellet was suspended in 30 mL of ice-cold column buffer containing 20 mM HEPES (pH 8.0), 500 mM NaCl and 1 mM EDTA. Cell breakage was performed by the addition of 3 g alumina and sonication (Fisher Model 550

Sonic Dismembrator) on ice for 15 sec followed by 20 sec cooling for 12 cycles. The sonicated crude extract was clarified by centrifugation at 12,000 x g for 30 min at 4°C. All subsequent work was carried out at 4°C. Isolation of the fusion protein was achieved by passing the clarified supernatant through a column containing chitin-coated beads (New England BioLabs). The column was washed with 150 column volumes of column buffer at 2 mL/min. On-column cleavage was achieved by passing 3 column volumes of column buffer containing 100 mM DTT. After 72 h, the target protein was eluted from the column with 2 bed volumes of column buffer. The eluted protein was precipitated by ammonium sulfate added to 75% saturation at 4°C. After centrifugation for 60 min at 17,000 x g, the pellet was suspended in 0.75 mL of 25 mM Bicine buffer, pH 8 and 5 mM DTT to protect the enzyme from oxidative damage. Excess salts were removed using a Hitrap 5 mL desalting column (Amersham Pharmacia) equilibrated with 25 mM Bicine (pH 8) and 5 mM DTT. The final volume of the desalted protein was approximately 2 mL, which was aliquoted in 25 µL volumes into sterile tubes and stored at -20°C. Enzyme activity was retained during storage under these conditions for at least 6 weeks.

4.2.3 Enzyme Activity Assays: Temperature and Substrate Concentration Effects

Wildtype and mutant PcpC activity was assayed by following the disappearance of the TCHQ substrate as described previously (Habash et al. 2002). The standard assay buffer contained 25 mM Bicine (pH 8.7), 10 mM ascorbic acid, 1 mM EDTA, 25 mM DTT, 4 mM reduced glutathione (GSH), and 100 µM TCHQ. Briefly, frozen protein stocks were thawed on ice and diluted 6-10-fold in assay buffer, depending upon the concentration of the protein stock solution. Protein concentration was determined using a

Coomassie Reagent assay (Pierce, Rockford, Illinois, USA) prior to each experiment. A TCHQ stock solution was prepared in assay buffer and diluted to 100 μ M for use in most experiments.

For experiments examining the effect of assay temperature on enzyme activity 1.2 mL of standard assay buffer was warmed to the assay temperature, 23 or 50°C. The reaction was initiated by the addition of ice-cold enzyme (wildtype or mutant PcpC). The concentration of enzyme added was adjusted based on preliminary activity experiments, which ranged from 4.5 – 36 nM. The volume of the enzyme added (< 1% of the total assay volume) did not significantly alter the temperature of the assay mix.

The effects of varying GSH (0.125 - 4 mM) and TCHQ (15 - 200 μ M) concentrations on enzyme activity were examined by changing the concentration of each test compound, while keeping all other conditions unchanged. One (1) mL of assay buffer containing the appropriate TCHQ or GSH concentration was warmed to 50°C, and similar to the temperature experiments, the reaction was initiated by the addition of ice-cold enzyme (wildtype or mutant PcpC). The concentration of enzyme added was adjusted based on preliminary activity experiments. For TCHQ concentration range experiments 4.5 nM of enzyme and for GSH concentration range experiments 9 nM enzyme were used. The volume of the enzyme added (< 1% of the total assay volume) did not significantly alter the temperature of the assay mix.

For all experiments, 50 μ L aliquots were removed, at specific time points, from the assay mix and added to an equal volume of 1 N HCl to stop the reaction. The HCl solution contained 180 μ M *p*-nitrophenol (Sigma) that served as an internal standard for subsequent quantification of substrate and products. Samples were centrifuged at 14,000

x g for 30 sec and analyzed by HPLC using a Hypersil Green ENV column (150 mm long x 4.6 mm inner diameter) at 22°C. The substrate (TCHQ), internal standard (*p*-nitrophenol), and products were eluted by acetonitrile:water (0.325:0.675, v/v) flowing at 0.75 mL/min. TCHQ loss was estimated by comparison of peak area relative to known quantities of authentic TCHQ standard (Sigma) prepared for each set of experiments. One unit (U) of enzyme activity was defined as the amount of enzyme that degraded 1 nmol of TCHQ per min.

All experiments were done in triplicate. Where applicable, results were subjected to Analysis of Variance (ANOVA). Statistically different results were considered to have $p \leq 0.05$.

4.2.4 Chemical Modification Studies

Chemical modification of PcpC wildtype and His variants using diethylpyrocarbonate (DEPC) was carried out at 23°C as described (Webb and Lee 1992). The standard assay buffer was modified during the chemical modification such that no GSH or TCHQ was present and the pH was maintained at 7 to reduce the likelihood that amino acids other than His were modified, but was otherwise the same as the standard assay buffer utilized for the enzyme activity assays. In addition, the protein stocks were diluted to the same concentration prior to the addition of DEPC. At various times, samples were removed from the incubation mixture for determination of residual enzyme activity, as described previously, at 50°C using the standard assay buffer at pH 8.7 containing GSH and TCHQ. The substrate (TCHQ) and cofactor (GSH) were tested for the ability to protect the wildtype PcpC from DEPC inactivation. In these experiments,

the wildtype PcpC was diluted in standard assay buffer containing either GSH (4 mM) or TCHQ (100 μ M), at pH 7, for 30 min at 4°C. Subsequent chemical modification was carried out at 23°C using 10 mM DEPC for 15 min. Enzyme activities were assayed as described previously at 50°C using the standard assay buffer at pH 8.7 with GSH and TCHQ (Habash et al. 2002). Appropriate controls were run concurrently without DEPC for all chemical modification studies.

4.2.5 Circular Dichroism (CD) Spectroscopy

For secondary and tertiary structure prediction protein samples were prepared by performing a buffer exchange into 5 mM Tris containing 5 mM DTT at pH 8.7. For secondary structure analyses a protein concentration ranging from 0.05 to 0.15 mg/mL was utilized. For tertiary structure prediction a protein concentration ranging from 0.5 - 1.5 mg/mL was utilized. Appropriate buffer blanks were also prepared. Protein samples and blanks were centrifuged and degassed prior to analysis. For secondary structure analyses in the far UV (250-185 nm), samples were placed in a quartz cuvette with a 0.1 cm path length. For tertiary structure analyses in the near UV (320-250 nm), samples were placed in a quartz cuvette with a 1 cm path length. Each sample was scanned four times at room temperature under continuous nitrogen flush with a Jasco 600 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The circular dichroism data were converted to molar ellipticity (θ).

4.3 Results and Discussion

In this study, the role of some His and Lys residues of the *Sphingomonas* sp. UG30 PcpC were characterized through site-directed mutagenesis and chemical modification. Our studies were prompted by sequence comparison data (Habash et al. 2002), which showed that His113 and Lys114 of our Zeta class PcpC are conserved and align with the corresponding His106 and Lys107 of the *P. mirabilis* Beta class GST sequence, which have been shown to be involved in GSH binding in the active site (Allocati et al. 2000). In addition, His113 of the UG30 PcpC aligns with His106 of the *E. coli* Beta class GST, which has been proposed to be involved in stabilization of the GSH thiolate ion (Nishida et al. 1998). Our previous study of essential amino acids present at the N-terminus of PcpC indicated a lack of a definitive N-terminal amino acid that appears to be involved with the stabilization of the GSH thiolate in the active site of PcpC (see Chapter 3). This is a role typically undertaken by an N-terminal serine in the Zeta class (Sheehan et al. 2001), to which PcpC has been designated (Anandarajah et al. 2000). Hence, histidine residues in PcpC have the potential for many different roles in catalysis.

The preliminary studies examining the impact of His/Lys residues upon wildtype PcpC activity involved chemical modification with diethyl pyrocarbonate (DEPC), a known histidine modifier. Chemical modification of PcpC with DEPC was made at pH 7. DEPC can modify amino acids other than histidine (including lysine residues); however, at neutral pH histidines are predominantly modified. Results indicated that PcpC activity decreased with increasing DEPC concentration (see Appendix 5). For future chemical modification experiments 10 mM DEPC was selected, based on its substantial impact on PcpC activity without completely inactivating the enzyme. Since

DEPC is a general modifier, site-directed mutagenesis of the previously created plasmid containing the *pcpC* gene (Chapter 2) was used to create five mutants, corresponding to four histidine residues and a lysine residue. Sequencing analyses using vector specific primers confirmed the single base change in each of the mutants. Furthermore, sequencing analyses indicated no other changes occurred in the *pcpC* gene. Each mutant was expressed in *E. coli* and purified as previously described (Habash et al. 2002). Typical yields for the wildtype and mutants were approximately 2 mg purified protein/L of culture. SDS-PAGE analyses indicated the proteins were purified to homogeneity with the expected molecular weight of 28.2 kDa (see Appendix 4).

Our initial hypothesis suggests that His113 and Lys114 were the best candidates for involvement in PcpC activity. The Lys114Ala mutant displayed an 8.3-fold increase in K_m for GSH. All other kinetic parameters remain unchanged relative to the wildtype PcpC (Table 4.2). The meaning of this is unclear, as the GSH binding domain is believed to be localized to the N-terminus of the enzyme. The His113Ala mutant showed only minor changes from the wildtype (Table 4.2). The specific activity of His113Ala mutant was 0.8-fold lower at 23°C and 1.8-fold higher at 50°C than the wildtype PcpC. As well, the k_{cat} and $K_{m,TCHQ}$ were 2.2-fold lower and 1.2-fold higher, respectively, than the wildtype. The $K_{m,GSH}$ was 1.6-fold higher than the wildtype. Thus, the kinetic parameters of His113Ala were not different from the wildtype, suggesting the involvement of other His residues in the DEPC inactivation.

Examination of the remaining His mutants indicated that all showed a decreased k_{cat} , for TCHQ and increased K_m values for TCHQ and GSH (Table 4.2). Of the $k_{cat}/K_{m,TCHQ}$ values, the His154Ala mutant showed the greatest (12.5-fold) decrease. The

$K_{m,GSH}$ also indicated a change from the wildtype with an increase of 8.9-fold. In addition, the His154Ala mutant was the only His mutant to have decreased specific activity at both 23 and 50°C, relative to the wildtype (retaining approximately 50% of wildtype activity). These results suggest that His154 may play a role in catalysis or in maintaining the proper PcpC three-dimensional conformation.

Chemical modification with DEPC of the His mutants determined if any of the mutants responded differently from the wildtype enzyme to chemical modification. Of the His mutants, His154Ala retained the highest residual activity (16.7%) on DEPC inactivation, all other mutants and the wildtype retained less than 10% of initial activity on DEPC addition. Nevertheless, the high degree of inactivation (greater than 83%) in all cases suggests that none of the His residues was solely responsible for the DEPC inactivation.

Protection studies of the wildtype enzyme with DEPC indicated that the presence of GSH or TCHQ did not alter the ability of DEPC to inhibit PcpC relative to the wildtype enzyme (see Appendix 6). In addition, as the concentration of DEPC was increased (from 0 to 15 mM DEPC) and all other components held constant, the level of inactivation increased for the PcpC wildtype (see Appendix 5). This suggests that none of the His residues reside near the substrate- or cofactor-binding sites. Furthermore, it implies that the large effect of the His154Ala mutation is the result of other as-yet-undefined role of His154 in maintaining the enzyme structure/conformation, rather than the possibility that this residue is involved in catalysis.

Table 4.2. Kinetic parameters of TCHQ degradation by PcpC wildtype and histidine and lysine mutants at 50°C. Results are averages and standard errors from at least three independent determinations.

Protein	Specific Activity		K_{cat} (s^{-1})	K_m , TCHQ (μM)	K_m , GSH (mM)	K_{cat}/K_m , TCHQ ($10^5 s^{-1} M^{-1}$)
	23°C	50°C				
Wildtype ^a	14.77	27.95 $\pm$ 2.3	42 $\pm$ 0.9	17.4 $\pm$ 0.7	149 $\pm$ 21.7	24.1
His113Ala	12.60 $\pm$ 1.1	51.91 $\pm$ 4	21 $\pm$ 1.7	23.7 $\pm$ 7.4	240 $\pm$ 33	8.9
Lys114Ala	11.58 $\pm$ 1.2	48.36 $\pm$ 4.9	19 $\pm$ 2.4	27 $\pm$ 10.8	1,233 $\pm$ 252	7.0
His148Ala	10.59 $\pm$ 1.1	41.96 $\pm$ 4.7	14.7 $\pm$ 0.9	32.5 $\pm$ 3.3	238 $\pm$ 29	4.5
His154Ala	5.49 $\pm$ 0.1	14.51 $\pm$ 0.5	9.3 $\pm$ 1.3	48.1 $\pm$ 11.5	1,318 $\pm$ 353	1.9
His181Ala	10.94 $\pm$ 2	39.39 $\pm$ 5.3	26.4 $\pm$ 2.7	12 $\pm$ 3.1	289 $\pm$ 100	22

^a k_{cat} and K_m results for the wildtype were obtained from (Habash et al. 2002)

Circular dichroism (CD) of the purified recombinant wildtype and mutant enzymes were studied to determine if the mutations introduced in PcpC resulted in major secondary or tertiary structural changes. Figure 4.1a presents the far UV CD data for the wildtype and mutant enzymes. Some deviation from the wildtype spectrum is shown for each of the mutants. For the near UV CD spectra, two distinct patterns appear (Figure 4.1b). Mutants His154Ala and His181Ala share a similar pattern and the wildtype and the three other mutants share another, but common pattern. The primary location of change appears to be a loss of some fine structure in the 260-280 nm region. This may be attributable to tertiary structure changes in PcpC because of the mutation of His to Ala resulting in the replacement of the bulky imidazole sidechain with a hydrogen. This could result in greater movement of the neighbouring Tyr, Phe, or Trp sidechains, which would appear as a change in the near UV CD spectrum between 260-280 nm. Thus, these results are indicative of a change in tertiary structure for His154Ala and His181Ala (Figure 4.1b). Comparing these results to previous studies in which CD was utilized to monitor structural changes of site-directed mutants relative to a wildtype enzyme (Caccuri et al. 2002), however, indicates that no major secondary or tertiary structural changes occurred in the PcpC His and Lys mutants. In addition, the CD results verified that the PcpC variants were not misfolded based on their similarity to the wildtype enzyme.

A potential explanation for the contrasting protection study results and site directed mutagenesis of Lys114 and His154 may be a result of the loose binding of GSH coupled with the formation of a weak pre-ternary complex in the PcpC active site. The formation of a covalent bond between GSH and the GST enzymes has been shown for some of the

GST classes, with the best example arising from the Beta class GSTs. The 3-D structure of the *P. mirabilis* GST has revealed that a mixed disulfide is formed between Cys10 within the enzyme active site and GSH (Rossjohn et al. 1998). In addition, the PcpC from *S. chlorophenolicum* ATCC 39723 has been shown to form a GSH-enzyme adduct via Cys13 that occurs only in the presence of TCHQ (McCarthy et al. 1997). Adduct formation in the ATCC PcpC is necessary for proper product formation (McCarthy et al. 1997). Our study of PcpC N-terminal mutants has shown that Cys14 plays an important role in enzyme activity by aiding in proper conformation of GSH in the active site (see Chapter 3); however, Cys14 neither forms a direct bond with GSH nor appears to be necessary for proper product formation. One potentially common feature between the two PcpC enzymes is the requirement for both GSH and TCHQ in the active site for proper formation of an enzyme-substrate complex. The formation of a weakly bound enzyme-GSH pre-complex has been shown for other GSTs (Caccuri et al. 1999, Neuefeind et al. 1997). Thus, unless both GSH and TCHQ are present in the active site neither may be held very tightly and as the DEPC concentration increases, its ability to displace GSH from more active sites might increase resulting in greater inhibition in total.

The impact of DEPC on PcpC activity could also have detrimental effects on the internal flexibility of the enzyme. Studies of the human Theta and Pi class GSTS have indicated that enzyme flexibility plays a strong role in the reaction mechanism and in substrate binding (Caccuri et al. 1996, Caccuri et al. 2001, Caccuri et al. 2001, Lo Bello et al. 1998, Ricci et al. 1996). In the Pi class GST, Ricci et al. (1996) have shown that a conformational change of the ternary complex is the rate-limiting step prior to the actual

chemical event that occurs between GSH and 1-chloro-2,4-dinitrobenzene. Solvent viscosity experiments with the Theta and Pi class GSTs indicate that enzyme movement is important to the reaction mechanism (Caccuri et al. 1996, Caccuri et al. 2001, Ricci et al. 1996). This may also be the case for PcpC, and potentially other Zeta GSTs, since the Zeta and theta classes are related (Sheehan et al. 2001). Hence, as the enzyme becomes modified by DEPC, steric hindrance of internal movement of the enzyme could increase and result in a reduction in activity.

In summary, our results suggest that His154 and Lys114 may be involved in the orientation of GSH in the active site of PcpC. In addition, it appears that GSH is bound loosely in the active site and that this may be an attribute of other Zeta class GSTs. Several pieces of evidence point in this direction: the lack of protection by either GSH or TCHQ to enzyme inhibition by DEPC; the lack of a directly bound GSH to the enzyme and the indication that PcpC and another Zeta class enzyme appear to undergo a conformational shift that may occur during GSH or TCHQ binding (Board et al. 2003, Polekhina et al. 2001); and the need for internal movements of the enzyme during the binding of its substrates and co-substrate, which may be inhibited by modification of histidine residues by DEPC. Future studies of PcpC, and of the Zeta class GSTs, must still address the question of how, and if, the glutathione thiolate is stabilized in the active site since no definitive amino acid has been conclusively identified with this role.

Figure 1a. Examination of PcpC wildtype and His and Lys mutants secondary structure by far UV circular dichroism. **b.** Examination of PcpC wildtype and His and Lys mutants tertiary structure by near UV circular dichroism.

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CHAPTER FIVE

Homology Modelling of the Tetrachlorohydroquinone Reductive Dehalogenases from *Sphingomonas* sp. UG30 and *S. chlorophenolicum* ATCC 39723

5.1 Introduction

Protein homology models can be utilized for the initial assessment of the three dimensional structure of a protein, in the absence of X-ray crystallography or NMR studies. Currently, several website based servers are available to aid in protein fold recognition for creating a protein homology model (Bujnicki et al. 2001). One of the most sensitive is the 3D-PSSM (three dimensional position-specific scoring matrix) recognition fold server as determined by the Critical Assessment of Techniques for Protein Structure Prediction (CASP4) (Bates et al. 2001) and by LiveBench-2, an automated evaluation of protein structure prediction servers (Bujnicki et al. 2001). The method to determine protein fold matches of the target protein sequence of unknown structure involves combining the results of several methods including: the creation of structural alignments of homologous proteins using the structural classification of proteins (SCOP); the creation of multiple alignments of sequences obtained by standard sequence searches; incorporating secondary structure matching and solvation potentials; and a descriptive keyword based system to further aid in matching proteins of known structure with the target sequence (Bates et al. 2001, Kelley et al. 2000).

There are many examples of GSTs where homology modeling has been used. Some relevant examples involve molecular modeling to supplement crystallography studies to aid in determining potential amino acids for site directed mutagenesis in

several different classes of GST including the Tau (Dixon et al. 2003), Pi (Lo Bello et al. 1998, Lo Bello et al. 2001), Mu (Ridder et al. 2002), Delta (Kettermann et al. 2001), and Theta (Flanagan et al. 1999) classes. In addition, homology modeling has been used for predictive modeling in the absence of a protein crystal structure in a murine alpha GST (Xia et al. 1999).

As no three-dimensional structure had been resolved for either UG30 or ATCC PcpC, simplistic homology models were created to help examine the results presented in this thesis. To the best of our knowledge these are the first models presented for the PcpC enzymes originating from *S. chlorophenolicum* ATCC 39723 and *Sphingomonas* sp. UG30. The creation of the models can help us understand how both of the wildtype PcpC enzymes function. In addition, information can be derived on how the mutations may have affected the UG30 enzyme, thereby providing some explanation for the observed changes in activity. Also, comparison of the two models may elucidate the reasons for the varied functional and kinetic differences observed between these two highly homologous proteins. It must be stressed that these are theoretical models and their use is simply to help provide a potential explanation of the mechanism of PcpC. The steps for deriving the homology models of the UG30 and ATCC PcpCs included template selection, template-target alignment, model building, and energy minimization.

5.2 Template Selection for *Sphingomonas* sp. UG30 and *S. chlorophenolicum* ATCC 39723 PcpCs

To select the template, the entire UG30 PcpC amino acid sequence was submitted to the 3D-PSSM website (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>), a web-based protein

fold recognition server (Kelley et al. 2000). Results obtained from the website provided the twenty top matches to the full length UG30 PcpC sequence. Selection of the template involved examining several criteria: E-value score, percent identity, and protein type. The E-value score represents an “expectation value” of the match. A score closer to zero represents a stronger match. 75% of the matches had E-value scores ≤ 0.05 indicating $\geq 95\%$ confidence of the match. The strongest match had an E-value score three orders of magnitude better than the next closest match, 4.01×10^{-6} and 0.00339, respectively. The strongest match also has an amino acid sequence with 24% identity to the UG30 sequence. In addition, the protein type of the strongest match was the apo form of a Phi class GST present in maize (previously known as a Type III-GST (Neuefeind et al. 1997)). Similar to PcpC, the maize GST is a homodimer and each subunit has a MW of 23.4 kDa (Neuefeind et al. 1997). Also, similar to PcpC, the Phi class GST conjugates GSH to its substrates, which are hydrophobic herbicides (Neuefeind et al. 1997). Thus, the target possesses many traits in common with PcpC and was utilized as a starting point for developing a homology model of the UG30 PcpC.

The amino acid sequence for the *S. chlorophenolicum* ATCC 39723 PcpC was also submitted to the 3D-PSSM website (Kelley et al. 2000). The results obtained were consistent with the top matches for UG30, i.e. the top match was the apo form of a Phi class GST present in maize, E-value score of 4.02×10^{-6} , and 24% amino acid sequence identity.

5.3 Template-Target alignment and Model building for the UG30 PcpC

A pairwise alignment of the template and target amino acid sequences was produced by the 3D-PSSM server (Figure 5.1) (Kelley et al. 2000). This alignment was manually adjusted to account for some difficulties with the 3D-PSSM alignment. The first eighty amino acids aligned with the template very well, as was expected given that they represent the glutathione-binding region for the GSTs, which is highly conserved among the various classes of GSTs. Although one deletion was needed at Gly37 from the UG30 sequence, this amino acid was located in a loop region as identified in the template sequence. Thus, its removal should not influence the model. Further manual adjustment of the alignment was necessary for the template sequence beyond Gly80 since the helix AAKLEV...LVRPLL (residues 91-123 of the Maize Phi GST) had been broken into three sections (see Figure 5.1). These sections were placed back together and, along with the short loop region (ASEFTDLL), were aligned with the ITYGH...DRIV (residues 109-152 of the UG30 PcpC) section of the UG30 sequence. This introduces a single deletion of 27 amino acids in the UG30 sequence that could not be accounted for by the template sequence and was left as a gap between Gly80 and Ile109 in the UG30 model. Secondary structure prediction posited that a coil (NVG...ADP) and helix (VENK...NFQV) (Kelley et al. 2000) were present in the deleted region (from Asn81 to Val108 in the UG30 sequence). The gap from UG30 in alignment near the C-terminus (between residues Thr237 and Ala238) was removed and substitutions of the template sequence continued to the end of the amino acid sequence. The final adjusted alignment is shown in Figure 5.2.

UG30_PcpC	MSEVSLNYT MSICSMKTRL AMEEFQVDYN DKQVDIGFAL ENFEEDYVRL	50
Maize phi GST	~APLKLYGMP LSPNVVRVAT VLNRKLLDFE IVEVDL~TTG AHKQPDFLAL	48
UG30_PcpC	NERAVVETLV VEDRVVTNEY NIVLEAAKLG ~~~~NVGIPA DPVENKAALD	96
Maize phi GST	NPFGQIEALV DGEELFESR AINRYIASKY ASEGTDLLPA TASA~AKLEV	97
UG30_PcpC	MFEGDQVNF QVITYGKGV ERGDELLIAR RERAREYAEK YPELRSIYQA	146
Maize phi GST	~L~~~~~ ~EVESEHFY ENASPLVF~ ~~~~~~ ~~~~~~	115
UG30_PcpC	AHDRIVEHGN CAYDADVVAQ AEIDQARLL ELD AHLADKP FIAGNTYSIA	196
Maize phi GST	~QLLVRPLLG GAPPDAVVDK HAECIAKVLD VYESHLARNK YLAGDEFTLA	164
UG30_PcpC	DIMWTVLEAR IEMLNMTAWI SERENLLQYY QRMKARRSFE T~~~ARVMEN	243
Maize phi GST	DANHASYLLY LSKTPKAGLV AAREHVKAHW EATVAPPAEQ KTVA~IPLLP	214
UG30_PcpC	WKGGI	248
Maize phi GST	PP	216

Figure 5.1 Partial sequence alignment created by 3D-PSSM server of the UG30 PcpC and the Maize Phi class GST. Note the helix sequence PATA...RPLL (residues 86- 123) of the Maize GST had been divided incorrectly into three sections.

UG30_PcpC	MSEVSLNYT MSICSMKTRL AMEEFQVDYN DKQVDIGFAL ENFEEDYVRL	50
Maize phi GST	~APLKLYGMP LSPNVVRVAT VLNRKLLDFE IVEVDL~TTG AHKQPDFLAL	48
UG30_PcpC	NERAVVETLV VEDRVVTNEY NIVLEAAKLG NVGIPADPVE NKAALDWFEK	100
Maize phi GST	NPFGQIEALV DGEELFESR AINRYIASKY ~~~~~~ ~~~~~~	78
UG30_PcpC	GDQVNFQVIT YEHKGVVERGD ELLIARRERA REYAEKYPEL RSIYQAAHDE	150
Maize phi GST	~~~~~AS ECTDLLPATA SAAKLEVWLE VESHHFYPNA SPLVFQLLVR	120
UG30_PcpC	IVEHGNCAYD ADVVAQAEID EQARLD ELDA HLADKPFIAG NTYSIADIMW	200
Maize phi GST	PLL~GGAPPD AAVVDKHAEO LAKVLLVYEA HLARNKYLAC DEFTLADANH	168
UG30_PcpC	TVLEARLEML NMTAWISERP NLLQYYQORMK ARRSTETARV MPNWKGGI	248
Maize phi GST	ASYLLYLSKT PRAGLVAAEP HVKSWWEAIV ARPAPQKTVA AIPLPPPP	216

Figure 5.2 The adjusted pairwise alignment used to incorporate the UG30 PcpC sequence into the template sequence from the Maize Phi class GST. Note the 27 amino acid deletion from the UG30 sequence between G80 and I109. The underlined areas represent α -helices from the Maize GST.

The homology model was created using SPDBV (version 3.7 (SP5)) (Guex and Peitsch 1997). Using the alignment in Figure 5.2, amino acid substitutions and the deletion of G37 were incorporated into the template sequence of the first eighty amino acids. Insertion of HG followed the helix PRGDEL...AHDRIVE (residues 117 – 153 of the UG30 sequence). Following the HG insertion the loop containing the amino model continued with substitutions outlined in the alignment present in Figure 5.2 to the end of the UG30 sequence. Four rounds of energy minimization were performed on the model. Energy minimization subjects each atom of the model through iterative force field calculations to minimize the forces acting upon each atom (Guex and Schwede 2001). This is a useful technique whenever a protein is distorted, such as after the introduction of amino acid substitutions that may not be in their “ideal” geometry (Guex and Schwede 2001).

It was observed that the HG insertion was not in an energetically favorable position. Therefore, the loop containing the amino acids EHGNC A was rebuilt using the Build Loop function of the SPDBV viewer to create a more favorable folding of the loop. Again, four rounds of energy minimization followed the loop building procedure.

A final adjustment of the model was performed by mutating each amino acid with itself. This step accounted for the fact that the optimal rotamers used during the initial substitution of the amino acids from the UG30 sequence into the template sequence were selected based upon surrounding amino acids from the template. Thus, by mutating each amino acid in the UG30 sequence the optimal amino acid conformation was selected based upon the surrounding amino acids that are from the UG30 sequence. This step was followed by four rounds of energy minimization.

5.4 Template-Target alignment and Model building for the *S. chlorophenolicum* ATCC 39723 PcpC

Similar to UG30, the alignment returned by 3D-PSSM between target (ATCC sequence) and template (Maize Phi GST) required some manual adjustment (Figure 5.3). The first eighty amino acids aligned with the template very well. Again, one deletion was needed at Gly37 from the ATCC sequence. As well, helix AAKLEV...LVRPLL (residues 91-123 of the Maize Phi GST) had been broken into three sections. These sections were placed back together, and along with the short loop region (ASEFTDLL), were aligned with the ITYGH...DRIV (residues 109-152 of the ATCC PcpC) section of the ATCC sequence. This introduces a single deletion of 27 amino acids in the ATCC sequence that could not be accounted for by the template sequence and was left as a gap between Gly80 and Ile109 in the ATCC model. Secondary structure prediction proposed that a coil (NVG...ADP) and helix (VENK...NFQV) (Kelley et al. 2000) were present in the deleted region. The gap from ATCC in alignment near the C-terminus was removed and substitutions of the template sequence continued to the end of the amino acid sequence. The final adjusted alignment is shown in Figure 5.4.

ATCC 39723 PcpC	MPEVSLNYT MSICSMKTRL AMEPEVVDYD DKQVIGFAL ENFEEDYVRL	50
Maize Phi GST	~APLKMGMP LSPNVVRVAT VLNEKELDFE IVPVLL~TG AHKQDFLAL	48
ATCC 39723 PcpC	NEKAVVETLV VEDRVVTNSY NIVLEAANVG ~~~~KVGIPA DEVENKAALD	96
Maize Phi GST	PPFGQIEALV DEDEVLFEER AINRYISKY ASEGTDLLPA TASA~AKLEV	97
ATCC 39723 PcpC	PFQKGDQVNF QVITYGKGV ERGDELLIAR REPAKEYAEK YPELRSTIYQA	146
Maize Phi GST	MLE~~~~~ ~~~VESHFY ENASPIVLFOL LVPPLL~~~~~	123
ATCC 39723 PcpC	AHDRIVEHGN CRYDADTAAQ AEVDTCRRLL ELDVHLADKP FIAGSNYSIE	196
Maize Phi GST	~~~~~G GAPDAAVNDK HAECARVLD VYEAHIAARNK YLAEDEFTLA	164
ATCC 39723 PcpC	IMWTVLEAR IEMLNMTAWI SEENLLYY QRMKARRSE T~~~SRVMEN	243
Maize Phi GST	DANHASYLY LSKTPKAGLV AAEHVKEWW EATVAPAFQ KTVAIPLEP	214
ATCC 39723 PcpC	WKGGI	248
Maize Phi GST	PP	216

Figure 5.3 Partial sequence alignment created by 3D-PSSM server of the ATCC PcpC and the Maize Phi class GST. Note the helix sequence PATA...RPLL (residues 86-123) of the Maize GST had been divided incorrectly into three sections.

ATCC 39723 PcpC	MPEVSLNYT MSICSMKTRL AMEPEVVDYD DKQVIGFAL ENFEEDYVRL	50
Maize Phi GST	~APLKMGMP LSPNVVRVAT VLNEKELDFE IVPVLL~TTG AHKQDFLAL	48
ATCC 39723 PcpC	NEKAVVETLV VEDRVVTNSY NIVLEAANVG KVGIPADPVE NKAALDWFQK	100
Maize Phi GST	PPFGQIEALV DEDEVLFEER AINRYISKY ~~~~~~	78
ATCC 39723 PcpC	GDQVNFQVIT YGHKGVERGD ELLIARRERA KEYAEKYPEL RSTIYQAAHDE	150
Maize Phi GST	~~~~~AS EGTDLLEATA SAAKLEWLE VESHHFYENA SPLVFLQLLR	120
ATCC 39723 PcpC	IVEHGNCYD ADTAAQAEVD ECRRLHLDV HLADKPFIAG SNYSIADIMW	200
Maize Phi GST	ELL~GGSPD LAVDKHAEQ LAHVLLVYEA HLRNKYLAE DEFTLADANH	168
ATCC 39723 PcpC	TVLLARTEML NMTAWISERP NLLYYORMK ARRSSETARV MPNWKGGI	248
Maize Phi GST	ASYLLYLSKT PKAGLVAARE HVKEWWEATV APAPFQKTVA AIPLPPPP	216

Figure 5.4 The adjusted pairwise alignment used to incorporate the ATCC PcpC sequence into the template sequence from the Maize Phi class GST. Note the 27 amino acid deletion from the ATCC sequence between G80 and I109. The underlined areas represent α -helices from the Maize GST.

The final alignment of the ATCC PcpC and the template sequence is identical to the UG30 PcpC and template alignment. Thus, the ATCC PcpC model was created using the UG30 model as a foundation. There are 14 amino acid differences between the UG30 and ATCC PcpCs, these were incorporated into the ATCC model from the UG30 model as follows: Ser2 → Pro, Asn30 → Asp, Arg53 → Lys, Lys78 → Asn, Leu79 → Val, Arg131 → Lys, Val163 → Thr, Ile169 → Val, Ala173 → Lys, Ala180 → Val, Asn190 → Ser, Thr192 → Asn. To note, two amino acid differences (amino acids 81 and 99) could not be incorporated into the ATCC model since they were part of the 27 deleted amino acids. Neither of these amino acid differences would be likely to significantly impact upon the model. The amino acid change from Asn81 in the UG30 PcpC to Lys in the ATCC PcpC would not impact the structure since this amino acid faces into the solvent, based upon the ATCC and UG30 models. With regard to the amino acid change from Gln99 in the UG30 PcpC to Asp, both amino acids are polar and hydrophilic in nature and thus would likely face into the solvent and would also likely not impact the structure of the model. Following the incorporation of the amino acid differences three rounds of energy minimization were carried out on the ATCC model returning its original energy level prior to the substitution of the amino acids outlined above.

5.5 Results and Discussion

5.5.1 Homology Model of UG30 PcpC

The final model contains 219 amino acids. Figures 5.5 and 5.6 present ribbon and molecular surface images, respectively, of the UG30 PcpC homology model. The model

of the UG30 PcpC possesses characteristics present in all classes of GST (Sheehan et al. 2001). In particular, the N-terminal region comprises a mixture of β -sheet and α -helices (amino acids 1-80), while the C-terminal region consists of α -helices (amino acids 117-248). The functional enzyme exists as a homodimer (not shown) and based on results from other Zeta class GSTs, we would expect the two PcpC subunits to join around a 2-fold rotational axis (Polekhina et al. 2001, Thom et al. 2001). Similar to the human Zeta class GST (Polekhina et al. 2001), α -helices 4 and 5 should form the contacts between the two subunits of the UG30 PcpC dimer.

The active site of the predicted UG30 model, as has been described for other GSTs, involves the ends of helices $\alpha 1$ and $\alpha 3$ at the N-terminal domain and $\alpha 4$ and $\alpha 6$ from the C-terminal domain (Figure 5.5). Interestingly, this includes the Ser12, Cys14, and Ser15 that have been studied in this thesis. Of particular note are Ser12 and Cys14, which line the floor of the GSH binding site and might be expected to participate in some aspects of catalysis. Ser15 points away from the active site and likely is not directly involved in catalysis. This is similar to the human and *A. thaliana* Zeta class GSTs, where the corresponding Ser residue also points away from the active site and is involved in H-bonding with surrounding residues (Polekhina et al. 2001, Thom et al. 2001). The UG30 model includes Pro57 (Pro56, represents the corresponding amino acid in the adjusted sequence), which is conserved amongst all GSTs. This Pro residue interacts by backbone H-bonding to the glutathione molecule (Thom et al. 2001). Also, the preceding residue, Val56 (Val55), may be involved with binding of GSH. This has been described for the human Zeta class GST where the backbone of the corresponding Val60 (Val60) interacts with the cysteinyl moiety of GSH in an anti-parallel fashion (Polekhina et al.

2001). The end of helix $\alpha 3$ contains several amino acids that may interact with the bound GSH including Ser69 (Ser68).

The proposed substrate-binding site, formed by the helices $\alpha 4$ and $\alpha 6$, is comprised mainly of hydrophobic amino acids facing the active site. This would provide a good environment for binding the hydrophobic substrate TCHQ.

Figure 5.5 Ribbon diagram of the UG30 PcpC homology model. Domain I consists of α -helices ($\alpha 1$ - $\alpha 3$) and β -sheets where the amino acids that define the glutathione binding site are located. Domain II comprises α -helices ($\alpha 4$ - $\alpha 10$) that contain amino acids that form the substrate binding site and the dimer interface.

Figure 5.6 Overlay of molecular surface and ribbon diagrams of the UG30 PcpC. Note the deep groove (indicated by black arrow on diagram) of the active site present in PcpC that is typical of GSTs.

5.5.2 Effect of N-terminal Site-Directed Mutants

An examination of the effect of the Ser12Ala, Cys14Ala, and Ser15Ala mutants using the UG30 homology model provides some insights on how these mutations may have affected UG30 PcpC activity (Figure 5.7). As expected for conservative substitutions of Ala either for Ser12, Ser15, or Cys14, there are no changes in the overall conformation of the backbone carbons for any of the mutants. One change that may occur involves disruption of H-bonds as a result of these mutations. Altering the H-bonding interaction could lead to a more thermolabile protein, as has been shown for the manganese- and iron-superoxide dismutases from *Escherichia coli* through mutation of a glutamine residue (Hunter et al. 2002).

The model of the wildtype UG30 PcpC indicates that Ser15 can H-bond with the –OH group of Thr18 and the amino group of Arg19 via its carboxyl group. The –OH side group of Ser15 forms a H-bond with the carboxyl group of Tyr9. Such H-bonding interactions are typical of Ser residues, i.e. the –OH side group H-bonds with the main chain atoms as has been shown previously for the human Zeta class GST (Board et al. 2003, Polekhina et al. 2001). Mutation of Ser15 to Ala results in the loss of H-bonding between Ser15 and Tyr9. This could promote the unfolding of the enzyme as β -sheet 1 loses contact with helix α 1. This effect may be exacerbated with an increase in temperature. This could also have been represented in the CD data, presented in Chapter 3 that indicated the loss of tertiary structure for the Ser15Ala mutant.

Figure 5.7a Ribbon diagram of the UG30 PcpC homology model with boxed area indicating the locations of Ser12, Cys14, and Ser15. **5.7b** Close up of boxed area in (a) of Ser12, Cys14, and Ser15 residues. Note that Ser 12 and Cys14 face into the active site, while the -OH of Ser15 faces away.

With regard to Ser12, the mutation to Ala did not change the H-bonding present in the wildtype enzyme, where main chain amino group of Ser12 H-bonds to the main chain carboxyl group of Tyr9. In addition, the carboxyl group of Ser12 is within H-bonding distance to the amino groups of Ser15 and Met16. The –OH group of Ser12 forms a H-bond with the amino group of Ser15. Thus, mutation of Ser12 to Ala appears to result in the loss of H-bonding to Tyr9. Since Ser12 is present at the terminus of α -helix 1 and Ser residues are important in capping the helix, the loss of the H-bond could result in destabilization of the helix structure and loss of thermostability.

There do not appear to be any H-bonds that are formed via Cys14. In addition, mutation to Ala does not change the bonding; however, the model does not provide any indication why the Cys14Ala mutant had a shift in temperature optimum relative to the wildtype PcpC enzyme.

Future experiments to examine other amino acids that could play roles in the catalytic mechanism of PcpC should include Ser69 (Ser68) and Tyr133 (Tyr104). Both of these –OH bearing amino acids face into the GSH binding site. These residues may be important since no definitive amino acid has been implicated in stabilization of the thiolate ion in the active site of PcpC.

5.5.3 Modeling the His and Lys Residues in UG30 PcpC

An inspection of the location of the four His residues present in the UG30 PcpC homology model provides some insight into the results obtained for the chemical modification experiments (Figure 5.8). The solvent exposed His113 (His84), His148 (His119) and His154 (His125) are such that chemical modification of these residues

could affect the ability of the UG30 PcpC to function properly through steric hindrance. This also supports the results of chemical modification of the various His mutants, such that more than one His residue may be near the active site as indicated by the DEPC inactivation of all mutants. An examination of the location of Lys114 (Lys85) in the homology model showed that it is located on a coil near the active site of the UG30 PcpC. It is possible that this region could move to allow the Lys residue to interact with the co-substrate, as demonstrated by an increase in K_M when this residue was mutated to Ala.

Figure 5.8 Ribbon diagram of UG30 PcpC homology model indicating locations of His (His113, His148, His154, and His181) and Lys (Lys114) residues.

5.5.4 Comparison of the ATCC and UG30 PcpC Models

Similar to the UG30 PcpC, the ATCC model is comprised of 219 amino acids. The two PcpC are built upon a common backbone, thus the general description of the ATCC model is the same as the UG30 model. Accordingly, the N-terminal region is formed by a mixture of α -helices and β -sheets. The C-terminal region contains only α -helices. The subunit interface for dimer formation is likely composed of α -helices 4 and 5.

None of the amino acid differences between the UG30 and ATCC PcpCs were present in the active site, which is comprised of the ends of helices $\alpha 1$ and $\alpha 3$ at the N-terminal domain forming the glutathione-binding site (Figure 5.9) and $\alpha 4$ and $\alpha 6$ from the C-terminal domain forming the substrate-binding site (Figure 5.10). Amino acids differences at residues 2, 30, 53, 78, 79, 163, 191, and 192 (residues 2, 30, 52, 77, 78, 134, 162, and 163, respectively in the truncated sequences) occurred at the periphery of the models. They appear to be exposed to solvent and, thus, would likely not cause deviations of the ATCC model from the UG30 model. Moreover the differences at residues 30, 53, 79, and 191 (residues 52, 78, and 162, respectively) were conservative changes between the two PcpC enzymes. When examining the amino acid differences at residues 131, 169, 173, and 180 (residues 102, 140, 144, and 151, respectively) we find them clustered near the proposed area of the dimer interface from the UG30 model (Figure 5.10). Interestingly, residues 169, 173, and 180 (residues 140, 144, and 151, respectively) correspond to the area of increased hydrophobicity in the UG30 sequence, relative to the ATCC sequence, as described in Chapter 2. The implications are that a stronger dimer interface could be formed in the UG30 PcpC. This may account for

tolerance of the UG30 PcpC for increased reaction temperatures and more alkaline pHs over the ATCC PcpC described in Chapter 2. Thus, the amino acids at positions 169, 173, and 180 (residues 140, 144, and 151, respectively in the truncated sequences) of the ATCC and UG30 models would be good targets for future studies examining the functional differences between the UG30 and ATCC PcpCs.

Figure 5.9 Ribbon diagram of the ATCC PcpC homology model of Domain I. The amino acid differences to the UG30 PcpC (Pro2, Asp30, Lys53, Asn78, and Val79) are indicated on the model as are Ser12 and Cys14 for reference to the glutathione-binding site.

Figure 5.10 Ribbon diagram of the ATCC PcpC homology model of Domain II. The amino acid differences to the UG30 PcpC (Lys131, Thr163, Val169, Lys173, Val180, Ser191, and Asn192) are indicated on the model. Note the location of residues Lys131, Val169, Lys173, and Val180 near the proposed dimer interface.

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CHAPTER SIX

Summary and Conclusions

6.1 Chapter Summaries

This thesis presents the experimental results of the characterization of the TCHQ reductive dehalogenase (PcpC) in *Sphingomonas* sp. UG30. The research proceeds from sequence analyses to cloning, protein purification, functional and kinetic characterization of the wildtype and site-directed mutant enzymes, chemical modification experiments, and metabolite detection using LC/MS. This research provides fundamental information on a bacterial reductive dechlorination enzyme, where the wildtype enzyme and site-directed mutants have been poorly characterized at the biochemical and physiological levels. The results may be useful for development of technology aimed at increasing the efficiency of biodegradation of toxic, highly chlorinated phenols.

Chapter 2 presents results of our initial characterization of the recombinant wildtype PcpC enzyme from UG30. Sequence analyses of the UG30 *pcpC* gene confirmed many similarities to the Zeta class GSTs, as had been shown previously for the *pcpC* gene from *S. chlorophenolicum* ATCC 39723 (Anandarajah et al. 2000). An alignment with bacterial GSTs of the Beta class further revealed conservation of His113 and Lys114 residues of the UG30 PcpC. The corresponding His106 and Lys107 have been shown to be essential in the *E. coli* and *P. mirabilis* GSTs. The alignment, therefore, suggests that His113 and Lys114 may play roles in the catalytic mechanism of the UG30 PcpC. The UG30 *pcpC* gene was cloned into an expression vector and

transformed into *E. coli*. PcpC was expressed and purified to homogeneity. The functional enzyme is a homodimer, with each monomer having a MW of ~28 kDa. The pH, temperature and dithiothreitol ranges and optima were determined. In addition, the kinetic parameters were determined for TCHQ and GSH. These results, in combination with previously published studies of important amino acids for GST activity, lay the groundwork for subsequent site-directed mutagenesis experiments.

Chapter 3 examines the role of several N-terminal amino acids (Tyr7, Tyr9, Ser12, Cys14, and Ser15) from the UG30 PcpC by site-directed mutagenesis. Mutations of Ser12 and Ser15 significantly decreased the reaction rate, however, their roles appeared to be more related to orientation of GSH in the active site rather than having direct roles in catalysis. Such a role has also been shown for the equivalent Ser14 residue in the human Zeta class GST, GSTZ1 (Board et al. 2003, Polekhina et al. 2001). Furthermore, mutation of N-terminal Tyr residues (Tyr7 and Tyr9) in PcpC, which have been implicated in the catalytic mechanism of many classes of GSTs, resulted in no change in activity. This is similar to the Beta class GSTs where studies have indicated the lack of a specific N-terminal amino acid involved directly in catalysis (Casalone et al. 1998, Nishida et al. 1994), and the typical role of an N-terminal Ser or Tyr is to aid in stabilization of the thiolate ion (Sheehan et al. 2001).

An important difference between the Beta and Zeta class GSTs, the latter of which includes the UG30 PcpC, appears to involve the role of the N-terminal Cys residue. In the Beta class GSTs, such as those from *P. mirabilis* and *E. coli*, the N-terminal Cys residue forms a direct link to the GSH through a disulfide bond (Caccuri et al. 2002, Nishida et al. 1998, Rossjohn et al. 1998). In contrast, the N-terminal Cys in

some Zeta class GSTs (Polekhina et al. 2001, Thom et al. 2001) and the UG30 PcpC, does not appear to form any such link. The Cys14Ala variant of the UG30 PcpC exhibited greatly decreased reaction rate, mainly due to a dramatic increase in K_m . In addition, the variant continued to form TriCHQ and DCHQ, in contrast to the PcpC from ATCC 39723 where the Cys13Ser variant was unable to carry out both dechlorination steps. Furthermore, the ATCC 39723 variant only formed glutathione conjugated TriCHQ or DCHQ depending upon whether TCHQ or TriCHQ was the initial substrate, respectively.

Chapter 4 examines the role of His and Lys residues on UG30 PcpC activity through chemical modification and site-directed mutagenesis. The role of His/Lys in the catalytic mechanism of the Zeta class GSTs had not been previously suggested. The lack of a definitive N-terminal amino acid that can aid in the catalytic mechanism of PcpC and a previous alignment with the GSTs from *P. mirabilis* and *E. coli* suggested the potential role of His and Lys residues in the catalytic mechanism of the UG30 PcpC. Our results showed that His residues and Lys114 are not involved directly in the catalytic mechanism of UG30 PcpC. Rather, our results suggest that H154 and K114 may be involved in the orientation of GSH in or near the active site. In addition, it appears that GSH is bound loosely in the active site, which was supported by two results in Chapter 3. First, GSH is apparently not directly bound to the enzyme through the formation of a disulfide bond with an N-terminal Cys. Second, PcpC may undergo a conformational shift during GSH or TCHQ binding, suggesting the substrate or co-substrate could be easily removed from the active site until the shift occurs. Future studies of PcpC, and of the Zeta class GSTs, must still address the question of how and/or if the glutathione thiolate is stabilized in the

active site since no definitive amino acid has been conclusively identified to play this role.

6.2 Comparison between the PcpC enzymes from *Sphingomonas* sp. UG30 and *S. chlorophenolicum* ATCC 39723

A theme presented throughout this thesis is the comparison of the two highly homologous (94% identity in primary sequence, 14 amino acid differences between the two enzymes) PcpCs from *Sphingomonas* sp. UG30 and from *S. chlorophenolicum* ATCC 39723. In the course of our studies several similarities and several important differences have come to light. The similarities between the two PcpC enzymes include: the requirement of GSH for the reaction to proceed; the use of a sulfhydryl reducing agent is critical to prevent enzyme inactivation due to modification of at least the N-terminal Cys; both wildtype enzymes transform TCHQ first to TriCHQ and then to DCHQ through reductive dehalogenation; both enzymes are homodimers of approximately the same MW; and both enzymes are likely members of the Zeta class of GST. These are general similarities characteristic of the GST enzyme family, to which both PcpCs are members. What has become very interesting is the growing list of differences between these two enzymes.

The functional and kinetic differences that have been uncovered through our experiments range from the general parameters for enzyme function to the role of specific amino acids involved in catalysis. As presented in Chapter 2, we found striking differences in the optimal temperature and optimum pH under which the two wildtype enzymes function. The UG30 PcpC had a pH optimum approximately 2 pH units higher

and a temperature optimum approximately 10°C higher than the *S. chlorophenolicum* PcpC. In addition, the UG30 PcpC was more efficient in its ability to turn over TCHQ. Chapter 2 also describes one of the most intriguing differences in that the UG30 PcpC does not exhibit substrate inhibition, contrasting the *S. chlorophenolicum* PcpC that has been reported to experience severe substrate inhibition (Anandarajah et al. 2000, Kiefer and Copley 2002a, Kiefer et al. 2002b). The reasons for the enhanced ability of the UG30 PcpC to catalyze the turnover of TCHQ to DCHQ as well as the differing reaction conditions are not known. One possibility may be related to the better accommodation of TCHQ at the substrate-binding region. Hydropathy plots have identified a slightly more hydrophobic region in or near the substrate-binding site in the UG30 PcpC, caused by three amino acid differences (Thr163→Val, Val169→Ile and Lys173→Ala) from the *S. chlorophenolicum* to the UG30 sequence. As well, there are three other amino acid differences between the enzymes that may play a role (Pro2 → Ser, Asp30 → Asn, Asn78 → Lys). Whether or not any of these amino acid differences are important for the differing activity of the UG30 PcpC remains to be determined.

Another difference involves the role of the N-terminal Cys present in both PcpCs. Studies with the *S. chlorophenolicum* PcpC have shown that the active site Cys13 is necessary for proper product formation (McCarthy et al. 1996, McCarthy et al. 1997). Although the reaction rate was unchanged, dead-end glutathione conjugated products dominated (GS-TriCHQ and GS-DCHQ) in catalysis of TCHQ by the Cys13Ser variant (McCarthy et al. 1996). In contrast, the Cys14Ala mutant of the UG30 PcpC exhibited reduced reaction rate; however, this mutant continued to form the normal products

TriCHQ and DCHQ, derived from the catalysis of TCHQ. The reason for this difference in the role of the N-terminal Cys is not known.

6.3 Future Studies to Examine Differences between the UG30 and ATCC 39723 PcpC enzymes.

The PcpC enzymes from UG30 and ATCC 39723 are highly homologous and differ in only fourteen amino acids in their primary sequences. Of interest in future studies is the identification of which amino acids are responsible for some of the differences between the two enzymes. Also of interest is to examine the isomerase activity the UG30 PcpC for comparison to the already established maleylacetoacetate isomerase activity of the *S. chlorophenolicum* PcpC (Anandarajah et al. 2000). As well, further data mining studies could identify new homologous proteins to PcpC, as new microbial genomic sequences are published on a continual basis. Lastly, studies should be undertaken to identify *Sphingomonas* sp. UG30 at the species level.

The above studies may help us understand how the two enzymes might have evolved their different characteristics. Of particular interest are those amino acids responsible for the differences in substrate inhibition, and optimal temperature and pH. As well, it will be of interest to identify the amino acids that are specifically required for the reaction mechanism of PcpC. Mutational studies of other Zeta class GSTs have not identified catalytically crucial amino acids. To date the mutation studies have uncovered amino acids linked to the orientation of substrate or co-substrate within the active site. Lastly, would be to examine if mutation of Cys158 could result in an enzyme that is not affected by oxygen, yet otherwise maintains normal enzyme functioning.

Of the fourteen amino acid differences between the PcpCs from *S. chlorophenolicum* ATCC 39723 and *Sphingomonas* sp. UG30, five are conservative and, therefore, not expected to drastically affect enzyme function and/or activity. This leaves nine amino acid differences, each with a potentially dramatic effect on enzyme activity. There are many unanswered questions that need to be addressed. For example, how did these differences happen so quickly, assuming the evolution of PcpC occurred after the introduction of PCP in the environment approximately 60 years ago? Also, why are there such differences in activity if both enzymes evolved in a similar fashion to catalyze the same reaction, i.e. the conversion of TCHQ to DCHQ? Further site-directed mutagenesis studies involving the nine different amino acids and/or structural studies are needed to resolve some of these questions.

An interesting avenue of future research for PcpC is in the area of directed evolution. The wildtype PcpC has been deemed an “immature enzyme” based on several characteristics, including the recent introduction of its principal substrate (TCHQ) in the environment, slow reaction rate, lower k_{cat}/K_m values suggesting an catalytically inefficient enzyme, and broad substrate range (Armstrong 1997, Copley 2000). Thus, further evolution of PcpC could further improve its efficiency, catalytic rate and/or the affinity for its substrate. Proteins can be improved by rational design whereby specific amino acid substitutions are incorporated on the basis of sequence comparison or 3-D structural information. This approach has limitations because most of the predicted changes occur in the active site or cofactor binding regions. Random approaches, such as directed evolution, can overcome this limitation and permit changes to occur throughout the primary amino acid sequence, as would typically occur in natural evolution. The

variants are selected by screening for the desired traits, for example faster reaction rate, better substrate affinity, or an expanded substrate range. Then, the variant's amino acid sequence can be determined to reveal the changes that have occurred in the enzyme.

Directed evolution has received much attention in the last few years because of its ability to generate "improved" enzymes and enzymatic pathways in the complete absence of any structural or functional information about the enzyme (Arnold and Volkov 1999, Arnold 2000, Arnold et al. 2001, Farinas et al. 2001, Wackett 1998). Several techniques have been developed to promote the creation of improved enzymes. The most widely used method is DNA shuffling (Stemmer 1994a, Stemmer 1994b), which involves the use of homologous variants of an enzyme or the development of enzyme variants using random or error-prone PCR. The genes encoding the variants are digested with DNase I. The fragments are then allowed to recombine and reassembled by PCR. The PCR products are cloned into *E. coli*, the protein expressed and screened for the desired enzyme properties.

With regard to the UG30 PcpC, desired improvements could include screening for an expanded substrate range. An interesting improvement in this regard would be to catalyze the additional dehalogenation of its product DCHQ to chlorohydroquinone and hydroquinone. With respect to the breakdown of PCP, only two enzymes would be necessary to dehalogenate PCP, as compared to the five that are currently present in the degradative pathway from *S. chlorophenolicum* ATCC 39723. Moreover, a general ability to dehalogenate a greater number of toxic halogenated compounds is always a beneficial trait, for example when dealing with a soil or water sample containing a number of contaminants. Functional changes to PcpC could include alteration of the

temperature optimum to a lower temperature; lowering of the pH optimum to neutral 7 rather than 8.7; and altering the inactivation of the enzyme that occurs in the absence of a reducing agent. Such changes to the PcpC enzyme would make it a better candidate for industrial applications because the environmental conditions necessary for optimal enzyme activity would be easier to maintain for bioremediation purposes. There are many examples of degradative enzymes that have taken advantage of DNA shuffling technology to create improved enzymes.

DNA shuffling of *bphA1* genes encoding biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400 resulted in an enzyme with enhanced degradation capacity for PCB and related biphenyl compounds (Kumamaru et al. 1998). In addition, the improved enzyme was able to degrade single aromatic hydrocarbons such as benzene and toluene, which were poor substrates for the original enzyme (Kumamaru et al. 1998). Directed evolution has been utilized on some GSTs to promote improved herbicide tolerance in plants using an improved Tau plant GST able to detoxify diphenylether herbicides such as fluorodifen (Dixon et al. 2003). These examples of improved enzymes for bioremediation purposes could be utilized for *in situ* and *ex situ* remediation of contaminated sites.

6.4 Structural Studies – The next logical step

Many protein studies, at some point, involve the determination of the 3-D structure of the protein via X-ray crystallography and/or nuclear magnetic resonance (NMR) studies. Such studies allow one to not only resolve the 3-D structure of an enzyme, but also identify crucial elements of an enzyme's reaction mechanism. With respect to PcpC, the

results of such studies can provide a number of important insights into its structure-function relationships. First, crystal structures of PcpC complexed with glutathione and TCHQ, separately, will show precisely which amino acid residues are important in cofactor and substrate binding as well as catalysis, and may provide a detailed picture of how the substrate interacts with the enzyme at the active site. Second, the structural information obtained may allow us to elucidate a catalytic mechanism for PcpC, which is still a matter of considerable speculation. Third, the structural information will provide a starting point for the rational design of site-specific mutants with altered substrate specificity and/or enhanced haloaromatic dehalogenation efficiency. Fourth, comparison of the structures of the substrate bound and substrate free forms of PcpC will characterize substrate-induced conformational changes that may occur, and reveal any structural inter-subunit communication which may occur between the active sites on different subunits.

To complement the crystallographic studies, NMR determinations should also be undertaken; however, PcpC is too large for 3-D structure determination by NMR. Therefore, such studies may have to be limited to specific domains of the enzyme. NMR can also be used to obtain data on substrate binding and kinetics from *in vitro* studies. For example, information about Cl⁻ binding and release can be assayed by ³⁵Cl NMR and this approach may prove useful for comparing enzyme variants.

Structural studies were attempted in collaboration with Dr. Hans Vogel's BioNMR group at the University of Calgary; however, several difficulties were encountered. Among these, the most intractable was the inability to grow crystals of PcpC. In

addition, it proved difficult to express the GSH binding domain of PcpC for structural studies by NMR. Despite these difficulties the structural studies are on-going.

6.5 Future Studies of Dehalogenases in Bioremediation

One of the applications of dehalogenases and the microorganisms that harbour them is the bioremediation of water and soil at sites contaminated with haloorganic compounds. Dehalogenases and microorganisms capable of mineralizing persistent toxic halogenated compounds to CO₂, H₂O and inorganic constituents provide an economical and safe mechanism to degrade toxic compounds without the introduction of toxic metabolites into the environment. Furthermore, molecular techniques, such as directed evolution, may allow individual enzymes or entire degradative enzymatic pathways to be refined to remain stable long-term and enhance their native capabilities to more quickly and more efficiently degrade toxic halogenated compounds.

Regarding *ex situ* bioremediation, a process that takes place in a bioreactor, for example, the ability to control the environmental conditions allows optimal bioavailability and degradation of the compound of interest. Dehalogenases such as PcpC can be utilized in two ways in an *ex situ* bioremediation process. First, an individual enzyme, such as PcpC, or an entire degradative pathway, such as the PCP degradation pathway, can be isolated and used. Second, whole cells can be utilized, either as the original strain or as a variant possessing enhanced enzymes or traits created by molecular techniques, such as DNA shuffling, to improve their use in bioremediation. The enzymes or cells can be used suspended in solution within a reactor; immobilized within or on top of a matrix, via adsorption or covalent bonds; or encased within gels or

microcapsules (Lopez et al. 2002, Park and Chang 2000). One area of particular interest involves the immobilization of individual enzymes or entire enzymatic pathways in an enzymatic membrane reactor (EMR). There are currently no studies documenting the use of EMRs in bioremediation of recalcitrant toxic halogenated compounds, however, EMR has been utilized for the enzymatic decolourization of dyes in effluents using manganese peroxidase from a white rot fungus (Lopez et al. 2002).

Ex situ bioremediation has several disadvantages. The greatest disadvantages of *ex situ* bioremediation are primarily related to the high cost of transporting contaminated soil or water to the bioremediation site and maintaining the bioremediation facility. In addition, to maintain constant high enzyme activity, it is necessary to add enzyme continuously as it becomes destabilized with time, as was shown for the decolourization of dyes by manganese peroxidase from a white rot fungus (Lopez et al. 2002). Another problem with a cell-free system is that some of the cofactors cannot be easily regenerated. As well, it is difficult and costly to provide an exogenous source of cofactor over long periods of time. This problem becomes more pronounced if many enzymes are involved, such as in the case of PCP degradation where five enzymes have been shown to be directly involved (Cai and Xun 2002, Dai et al. 2003). One solution is to use live cells that can regenerate the cofactors and the enzymes. An example such as *Sphingomonas* sp. UG30 can fulfill this role since it possesses the degradative pathway and thus would be able to regenerate the cofactors and enzymes for the degradation of PCP. These same characteristics also make *Sphingomonas* sp. UG30 an appealing candidate for *in situ* bioremediation

In situ bioremediation involves microorganisms that are either seeded at the site of contamination or the site is conditioned to encourage the growth of the microorganisms of interest, i.e. via biostimulation (Iwamoto and Nasu 2001). *In situ* remediation does not require transportation of the soil or water for clean up, there is less disruption of the environment, and is likely to be less costly. In addition, the utilization of an organism such as *Sphingomonas* sp. UG30 has the potential to overcome some of the limitations associated with *in situ* bioremediation. The principal difficulties with *in situ* remediation techniques are the bioavailability of the compound to be degraded, the presence of inhibitors of microbial and enzymatic activity, and introducing a new microorganism to an already established community.

In particular, having been isolated from a PCP-contaminated soil is an important characteristic that will likely permit *Sphingomonas* sp. UG30 to be established in other PCP-contaminated sites. In, for example, PCP contaminated soil the diversity of the original microbial community would be decreased, giving an introduced microorganism, such as *Sphingomonas* sp. UG30, a better chance at colonizing the soil. The introduced microorganisms are not required to compete with an established community at a contaminated site. This has been observed in remediation studies with *Sphingobium chlorophenolicum* strain RA2, a PCP-mineralizing soil organism related to *Sphingomonas* sp. UG30. Introduction of strain RA2 resulted in the colonization and mineralization of soil contaminated with varying concentrations of PCP (Colores and Schmidt 1999). In non-contaminated control soils, however, introduced *S. chlorophenolicum* RA2 was unable to compete with the existing soil microbiota and the

number of strain RA2 decreased to undetectable levels during the study (Colores and Schmidt 1999).

With the ability to introduce and maintain a microorganism capable of degrading the compound of interest, other modifications of the environment (for example, soil or groundwater amendments) can aid in increasing the bioavailability of the toxic compound as well as decreasing the levels of inhibitory compounds that could impact upon bioremediation. For example, the addition of organic matter or increasing the soil water content has been shown to increase PCP degradation by *S. chlorophenolicum* ATCC 39723, on the other hand anoxic conditions and elevated Cl^- levels inhibited PCP degradation (Seech et al. 1991). Although, we have only briefly examined the potential of *ex situ* and *in situ* remediation technologies there is much promise in their utilization for degradation of persistent toxic halogenated compounds.

Future analyses of whole genomes and DNA fragments coding for the reductive dehalogenases and related sequences will yield novel insight into the functioning, accessory proteins, and evolution of the reductive dehalogenases. We expect the study of bacterial reductive dehalogenases to broaden as new genera, species, and strains of anaerobic and aerobic microorganisms that produce not only reductive dehalogenases, but also various other dehalogenating enzymes are discovered. The knowledge gained from studying these enzymes will help us understand the evolutionary relationship between dehalogenases. It will also aid in the development of applications for dehalogenases in the remediation of environments contaminated with toxic halogenated organic compounds.

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Appendix 1

Sphingomonas sp. UG30	ATGCTCTGAAG TCAGTCTCTA TAATTATACC ATGTCGATCT GTTCGATGAA	50
S. chlorophenolicum	ATGCTCTGAAG TCAGTCTCTA TAATTATACC ATGTCGATCT GTTCGATGAA	50
Sphingomonas sp. UG30	SACCCGECTC GCGATGGAG AATTCGGGT CGACTATAC GACAAGCAGG	100
S. chlorophenolicum	SACCCGECTC GCGATGGAG AATTCGGGT CGACTATAC GACAAGCAGG	100
Sphingomonas sp. UG30	TGGAATTCGG TTTCGCCCT GAAAACCTCG AACCGACTA TGTTCGGCTG	150
S. chlorophenolicum	TGGAATTCGG TTTCGCCCT GAAAACCTCG AACCGACTA TGTTCGGCTG	150
Sphingomonas sp. UG30	AATGAAAGGG CGGTCGTGCC CACCTGGTC GTCGGCGATC GCGTCGTCAC	200
S. chlorophenolicum	AATGAAAGGG CGGTCGTGCC CACCTGGTC GTCGGCGATC GCGTCGTCAC	200
Sphingomonas sp. UG30	CAATAGCTAC AATATCGTCA TGGAGCGGCG AAGCTGGGC AATGTCGGCA	250
S. chlorophenolicum	CAATAGCTAC AATATCGTCA TGGAGCGGCG AAGCTGGGC AATGTCGGCA	250
Sphingomonas sp. UG30	TTCCGCCGA CCCGTCTGAG AACCAAGGCCG CGCTCGATTG GTTCCAGAAG	300
S. chlorophenolicum	TTCCGCCGA CCCGTCTGAG AACCAAGGCCG CGCTCGATTG GTTCCAGAAG	300
Sphingomonas sp. UG30	GGCGATCAGG TCAATTTCCA GGTCAATCACC TATGGACACA AGGGCGTGCC	350
S. chlorophenolicum	GGCGATCAGG TCAATTTCCA GGTCAATCACC TATGGACACA AGGGCGTGCC	350
Sphingomonas sp. UG30	GCGCGGGGAC GAACTGCTGA TCGCGCGCCG CGAACGCGCG AGGGAATATG	400
S. chlorophenolicum	GCGCGGGGAC GAACTGCTGA TCGCGCGCCG CGAACGCGCG AGGGAATATG	400
Sphingomonas sp. UG30	CCGATAAATA TCCCGAATG CGTCCATTT ACCAGGCCG GCACGATCGC	450
S. chlorophenolicum	CCGATAAATA TCCCGAATG CGTCCATTT ACCAGGCCG GCACGATCGC	450
Sphingomonas sp. UG30	ATCGTGGAGC ATGGCAATTG CGCCTATGAC GCGGACCTGG TGGCCAGGC	500
S. chlorophenolicum	ATCGTGGAGC ATGGCAATTG CGCCTATGAC GCGGACCTGG TGGCCAGGC	500
Sphingomonas sp. UG30	GGAAATCGAC CTGCAGGGGC GCTCTGACGA ACTGGACGCG CATCTGGCGG	550
S. chlorophenolicum	GGAAATCGAC CTGCAGGGGC GCTCTGACGA ACTGGACGCG CATCTGGCGG	550
Sphingomonas sp. UG30	ACAAGCCGTT CATCGCGGGC AATACGTACA GCATCGCCGA TATCATGTGG	600
S. chlorophenolicum	ACAAGCCGTT CATCGCGGGC AATACGTACA GCATCGCCGA TATCATGTGG	600
Sphingomonas sp. UG30	ACGGTCCTGC TGGCGCGGAT CGAATGCTC AACATGACGG CGTGGATCAG	650
S. chlorophenolicum	ACGGTCCTGC TGGCGCGGAT CGAATGCTC AACATGACGG CGTGGATCAG	650
Sphingomonas sp. UG30	CGAGCGTCCG AACCTGCTCG CTTATTATCA GCGGATGAAG GCGCGCCGCA	700
S. chlorophenolicum	CGAGCGTCCG AACCTGCTCG CTTATTATCA GCGGATGAAG GCGCGCCGCA	700
Sphingomonas sp. UG30	GCTTGAAAC GCGCGGGT ATGCCCAAT GGAAGGGCG CATCTGA	747
S. chlorophenolicum	GCTTGAAAC GCGCGGGT ATGCCCAAT GGAAGGGCG CATCTGA	747

Sequence alignment of the *Sphingomonas* sp. UG30 (Accession number: AY057901) and *S. chlorophenolicum* ATCC 39723 (Accession number: M98559) PcpC nucleic acid sequences. These amino acid sequences share 90% identity. Note the basepair differences between the two sequences are highlighted.

Appendix 2

<i>Sphingomonas</i> sp. UG30	M	E	V	S	L	Y	N	T	M	S	I	C	S	H	K	T	R	L	A	M	E	E	F	G	V	D	T	D	K	Q	V	D	I	G	F	A	L	E	N	F	E	P	D	Y	V	R	L	50			
<i>S. chlorophenolicum</i>	M	E	V	S	L	Y	N	T	M	S	I	C	S	H	K	T	R	L	A	M	E	E	F	G	V	D	T	D	K	Q	V	D	I	G	F	A	L	E	N	F	E	P	D	Y	V	R	L	50			
<i>Sphingomonas</i> sp. UG30	N	E	R	A	V	V	P	T	L	V	G	D	R	V	T	N	S	Y	N	I	V	L	E	A	A	M	G	E	V	G	I	P	A	D	P	V	E	N	K	A	A	L	D	W	F	E	K	100			
<i>S. chlorophenolicum</i>	N	E	R	A	V	V	P	T	L	V	G	D	R	V	T	N	S	Y	N	I	V	L	E	A	A	M	G	E	V	G	I	P	A	D	P	V	E	N	K	A	A	L	D	W	F	E	K	100			
<i>Sphingomonas</i> sp. UG30	G	D	Q	V	N	F	Q	V	I	T	Y	G	H	K	G	V	P	R	G	D	E	L	L	I	A	R	R	E	R	A	E	Y	A	E	K	Y	P	E	L	R	S	I	Y	Q	A	A	H	D	R	150	
<i>S. chlorophenolicum</i>	G	D	Q	V	N	F	Q	V	I	T	Y	G	H	K	G	V	P	R	G	D	E	L	L	I	A	R	R	E	R	A	E	Y	A	E	K	Y	P	E	L	R	S	I	Y	Q	A	A	H	D	R	150	
<i>Sphingomonas</i> sp. UG30	I	V	E	H	G	N	C	A	Y	D	A	D	V	A	Q	A	E	E	D	L	Q	E	R	L	D	E	L	D	E	H	L	A	D	K	P	F	I	A	G	E	N	T	Y	S	I	A	D	I	N	W	200
<i>S. chlorophenolicum</i>	I	V	E	H	G	N	C	A	Y	D	A	D	V	A	Q	A	E	E	D	L	Q	E	R	L	D	E	L	D	E	H	L	A	D	K	P	F	I	A	G	E	N	T	Y	S	I	A	D	I	N	W	200
<i>Sphingomonas</i> sp. UG30	T	V	L	L	A	R	I	E	M	L	N	M	T	A	W	I	S	E	R	P	N	L	L	A	Y	Y	Q	R	N	K	A	R	R	S	F	E	T	A	R	V	M	P	N	W	K	G	G	I	248		
<i>S. chlorophenolicum</i>	T	V	L	L	A	R	I	E	M	L	N	M	T	A	W	I	S	E	R	P	N	L	L	A	Y	Y	Q	R	N	K	A	R	R	S	F	E	T	A	R	V	M	P	N	W	K	G	G	I	248		

Sequence alignment of the *Sphingomonas* sp. UG30 (Accession number: AY057901) and *S. chlorophenolicum* ATCC 39723 (Accession number: M98559) PcpC amino acid sequences. These amino acid sequences share 94% identity. Note the 14 amino acid differences between the two sequences are highlighted.

Appendix 3

Representative SDS-PAGE of purified wildtype PcpC and N-terminal variants of PcpC. Lanes: 1 and 8: molecular weight protein standards (from top to bottom 97, 66, 45, 31, 21, and 15 kDa); Lane 2: wildtype PcpC; Lane 3: Tyr7Phe PcpC variant; Lane 4: Tyr9Phe PcpC variant; Lane 5 Ser12Ala PcpC variant; Lane 6 Cys14Ala PcpC variant; and Lane 7 Ser15Ala PcpC variant. Lanes 2-7 each contains 1 μ g/mL protein.

Appendix 4

Representative SDS-PAGE of purified wildtype PcpC and His and Lys variants of PcpC. Lanes: 1 and 8: molecular weight protein standards (from top to bottom 97, 66, 45, 31, 21, and 15 kDa); Lane 2: wildtype PcpC; Lane 3: His113Ala PcpC variant; Lane 4: Lys114Ala PcpC variant; Lane 5 His148Ala PcpC variant; Lane 6 His154Ala PcpC variant; and Lane 7 His181Ala PcpC variant. Lanes 2-4, 6, and 7 each contains approximately 1 $\mu\text{g/mL}$ protein. Lane 5 contains 0.5 $\mu\text{g/mL}$ protein.

Appendix 5

Results of diethyl pyrocarbonate (DEPC) inactivation of PcpC wildtype enzyme. DEPC inactivation conditions of PcpC were made at pH 7 and 23°C. Enzyme assay reaction conditions: 25 mM Bicine (pH 8.7), 100 μM TCHQ, 4 mM GSH, 25 mM DTT, 12 mM ascorbic acid, 1 mM EDTA, and 1 μg/mL protein at 50°C. Results are averages of two independent determinations.

Appendix 6

Results of protection studies using tetrachlorohydroquinone (TCHQ) or glutathione (GSH) to prevent diethyl pyrocarbonate (DEPC) inactivation of PcpC wildtype enzyme. The wildtype PcpC was diluted in standard assay buffer containing either GSH (4 mM) or TCHQ (100 μ M), at pH 7, for 30 min at 4°C. Subsequent chemical modification was carried out at 23°C using various concentrations of DEPC for 15 min. Enzyme activities were assayed at 50°C using the standard assay buffer at pH 8.7 with GSH and TCHQ. Results are averages of two independent determinations.