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Shree Nath Singh *Editor*

# Microbial Degradation of Xenobiotics

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Editor

# Microbial Degradation of Xenobiotics

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Shree Nath Singh
Environmental Science Division
National Botanical Research Institute
Rana Pratap Marg
226001 Lucknow
India
e-mail: drsn06@gmail.com

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*In loving memory of my parents*

# Preface

Microorganisms are ubiquitous in the environment playing an important role in biogeochemical cycling. However, their ability to metabolize xenobiotic compounds has received much attention in recent years due to their environmental persistence and toxicity. Hence, microbial degradation of xenobiotics is, today, seen as both cost-effective and eco-friendly technology for removing these pollutants by a process known as bioremediation. Earlier researchers have confirmed that microbes are capable of degrading a wide range of organic pollutants. However, process of biodegradation is generally very slow and hence, this process may be accelerated by augmenting pure and mixed cultures of microorganisms in both aerobic and anaerobic conditions. Metabolic intermediates formed in the degradative pathways were also examined for their toxicity assessments using bacteria and higher organisms. Many of degradative genes responsible for xenobiotic metabolism are present on plasmids, transposons or are grouped in clusters on chromosomes. This indicates evolution of degradative pathways and makes the genetic manipulation easier. Development of the transgenic microbial strains highly capable of degrading xenobiotics is now possible through biotechnological approaches. Besides, several catabolic enzymes involved in xenobiotic metabolism have been isolated and characterized. A number of environmental factors, including pH, temperature, bioavailability, nutrient supply and oxygen availability have been shown to affect biodegradation process. These factors have to be optimized to obtain an effective microbial treatment process for the industrial organic wastes at bench and pilot scales. However, in the field scale treatment, all environmental factors cannot be manipulated to enhance the degradation process.

To update the knowledge on bioremediation which is a natural attenuation process, I present before you an edited volume on 'Microbial degradation of xenobiotics' which has focused on different aspects of microbial degradation of xenobiotic compounds, like poly aromatics hydrocarbons, polychlorophenols, polyurethane, dye containing wastewater, water soluble polymers, azo dyes, explosives, chloroorganic pollutants, styrene, trinitrophenol and high molecular weight alkanes. These aspects have been discussed in 17 chapters contributed by the leading scientists drawn from all over the world.

In this endeavor, I am not alone, but assisted by many fellow workers. First of all, I would like to acknowledge all the contributors who responded to my request and very enthusiastically contributed their chapters containing the latest developments on the relevant issues. The services rendered by my own research scholars Mrs. Babita Kumari, Ms. Shweta Mishra, and Mrs. Sadhna Tiwari in this endeavor are remarkable and highly appreciable. Besides, laboratory trainees Ms. Namarata Pandey, Ms. Jyoti, Ms. Rashi Singhal, Ms. Deepika Verma, Ms. Radha Verma, Ms. Shilpi Dupey and Ms. Shilpi Kumari are also duly acknowledged for their multifaceted help and support. Mr. Dilip Chakraborty deserves special appreciation for computer work for preparing the manuscript on the book format.

Lastly, I express my sincere thanks to my family members Mrs. Manorma Singh (wife), Dr. Ragini Singh (daughter), Mr. Pritish Kumar Singh (son) and the little champ Antra for their inspiration, endurance and moral boost up in this endeavor.

S. N. Singh

# Contents

# Contributors

**R. Balasubramanian** Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore

**Anat Bernstein** Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel

**Tomáš Cajthaml** Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic, e-mail: cajthaml@biomed.cas.cz

**Pritam Chattopadhyay** Plant Biotechnology Laboratory, Department of Botany, Visva-Bharati, Santiniketan, India

**Indrani Ghosh** Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

**Gary T. Howard** Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402, USA, e-mail: ghoward@selu.edu

**Leela Iyengar** Centre for Post graduate Studies, Jain University, Bangalore, India

**Stefan R. Kaschabek** Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany

**Fusako Kawai** Center for Nanomaterials and Devices, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, e-mail: fkawai@kit.ac.jp

**Christian Kennes** Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain, e-mail: kennes@udc.es

**B. Kumari** Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

**Toshinari Maeda** Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan, e-mail: toshi.maeda@life.kyutech.ac.jp

**Ernest Marco-Urrea** Department of Chemical Engineering and Institute of Environmental Science and Technology, Autonomous University of Barcelona, 08193 Bellaterra, Spain

**S. Maruthamuthu** Corrosion Protection Division, Biocorrosion Group, Central Electrochemical Research Institute, Karaikudi 630 006, India

**Shweta Mishra** Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

**Pijush Kanti Mondal** Environmental Research laboratory, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, Uttar Pradesh 202 002, India, e-mail: pijushamu@gmail.com

**Suparna Mukherji** Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

**Hiroaki I. Ogawa** Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

**Anjali Pandey** Department of Chemistry, Indian Institute of Technology, Kanpur, India, e-mail: anjali@iitk.ac.in

**José A. Perales-Vargas-Machuca** Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain

**Pattanathu K. S. M. Rahman** Chemical and Bioprocess Engineering Group, School of Science and Engineering, Teesside University, Middlesbrough, Tees Valley, TS13BA, UK, e-mail: p.rahman@tees.ac.uk

**A. Rajasekar** Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore, e-mail: e-mail: esera@nus.edu.sg

Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore, e-mail: rajasekar.aruliah@gmail.com

**C. A. Reddy** Department of Microbiology and Molecular Genetics and The NSF Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-4320, USA, e-mail: reddy@msu.edu

**Elisa Rojo-Nieto** Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain, e-mail: elisa.rojo@uca.es

**Zeev Ronen** Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel, e-mail: zeevrone@bgu.ac.il

**Sukanta K. Sen** Microbiology Division, Department of Botany, Visva-Bharati, Santiniketan, India, e-mail: sksenvb@rediffmail.com

**Antaryami Singh** Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India

**Poonam Singh** Division of Toxicology, Central Drug Research Institute, Lucknow, India

**Rita Singh** Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India, e-mail: singhritadr@yahoo.com

**S. N. Singh** Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India, e-mail: drsn06@gmail.com

**Shelly Sinha** Rabindra Mahavidyalaya, Champadanga, Hooghly, West Bengal, India

**Kateřina Svobodová** Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

**Y. P. Ting** Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore

**Dirk Tischler** Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany, e-mail: Dirk-Tischler@email.de

**María C. Veiga** Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain

**Luying Xun** School of Molecular Biosciences, Washington State University, Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA, e-mail: xun@mail.wsu.edu

# Chapter 1
# Microbial Degradation of Polychlorophenols

**Luying Xun**

## 1.1 Introduction

Polychlorophenols are major environmental pollutants, and their degradation by microorganisms has been extensively studied for the purpose of bioremediation. Three different metabolic pathways for aerobic degradation of polychlorophenols have been completely worked out, revealing the metabolic diversity for these structurally similar compounds. Substituted quinols, rather than catechols, are key metabolic intermediates of polychlorophenol biodegradation. Substituted quinols and quinones are also called as *p*-hydroquinones and *p*-benzoquinones, reflecting the reduced and oxidized forms. For example, tetrachloroquinol is the same as tetrachloro-*p*-hydroquinone, and tetrachloroquinone is often referred as tetrachloro-*p*-benzoquinone. Characterization of individual enzymes has led to the discoveries of novel dechlorination mechanisms. The genes coding for the enzymes have been cloned and sequenced, and the gene organization and regulation suggest that recent gene recruitments have occurred for the degradation of some polychlorophenols.

### *1.1.1 Sources of Polychlorophenols*

Trichlorophenols can be naturally produced, but pentachlorophenol (PCP) is anthropogenic in origin. Hoekstra et al. (1999) have reported the production of 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4,5-trichlorophenol (2,4,5-TCP) as well as

L. Xun (✉)
School of Molecular Biosciences, Washington State University,
Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA
e-mail: xun@mail.wsu.edu

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less chlorinated phenols from spiked $Na^{37}Cl$ in soils of a Douglas fir forest. However, tetrachlorophenols and pentachlorophenol (PCP) are not produced from the spiked $^{37}Cl$ after one year in situ incubation. There is no evidence of natural production of PCP. PCP is manufactured either by phenol chlorination with chlorine gas or alkaline hydrolysis of hexachlorobenzene, producing a technical grade of PCP that contains other polychlorophenols as impurities (WHO 1987). Merz and Weith first synthesized PCP in 1872 (Merz and Weith 1872). The massive release of PCP into the environment is mainly associated with its use as a wood preservative, a practice starting in the 1930s (Crosby 1981). PCP-treated lumbers are commonly used for outdoor structures, but some have been used to build wine cellars and log houses. The vapors of polychlorophenols released from the building materials can contaminate wine, giving it a corky taste (Suckling et al. 1999), probably due to the formation of chloroanisoles (Coque et al. 2003). People living in PCP-treated log houses have elevated blood levels of PCP over control groups (Cline et al. 1989). Further, polychlorophenol derivatives are often used as herbicides and fungicides. 2,4,5-Trichlorophenoxyacetate (2,4,5-T), a derivative of 2,4,5-TCP, is a potent herbicide and is a major ingredient of "Agent Orange" used for defoliation during the Vietnam War in the 1960s (Firestone 1978). Prochloraz, a derivative of 2,4,6-TCP, is an effective fungicide for plant pathogens (Birchmore and Meneley 1979). Consequently, a wide usage of polychlorophenols and their derivatives have resulted in environmental contamination.

The main sources of polychlorophenol contamination are from their production, application and discharge. The previously uncontrolled disposal has resulted in a widespread contamination of polychlorophenols, e.g. at least 415 locations of former wood preserving facilities are contaminated with polychlorophenols (Middaugh et al. 1994). Their hazardous nature has promoted many countries to regulate their use. In the United States, the release of polychlorophenols requires registration with the Environmental Protection Agency, and the data are published in Toxic Release Inventory: Public Data Release (EPA 2006).

### *1.1.2 Toxicity of Polychlorophenols*

Polychlorophenols are notorious for several reasons. First, they are harmful to all life forms because they disrupt the integrity and function of biological membranes (Cunarro and Weiner 1975; Escher et al. 1996). Second, their metabolites are also toxic. Human uptake of polychlorophenols is rapid via three mechanisms: skin absorption, inhalation, and ingestion (WHO 1986; Proudfoot 2003). High dose leads to hyperthermia, convulsions, and rapid death. The effects of low dose are unclear, resulting in elevated blood chlorophenol levels, which can be metabolized to chloroquinols or conjugated to polychlorophenol glucuronides for renal excretion (Uhl et al. 1986). The oxidation of chloroquinols and reduction of chloroquinones lead to the formation of reactive oxygen species, causing DNA damage (Dahlhaus et al. 1995) and other oxidative stresses (Wang et al. 2001).

Third, technical-grade polyclorophenols contain impurities, e.g. chlorinated dibenzo-*p*-dioxins and dibenzofurans, which are highly carcinogenic (Firestone 1978; Kaiser 2000). They are produced from polychlorophenols during manufacturing processes (Crosby 1981), and they can also be formed via biotransformation in soils (Hoekstra et al. 1999).

## 1.2 Microbial Degradation of Polychlorophenols

The most efficient and economical approach to the removal of low concentrations of polychlorophenols from contaminated soils and aquifers is bioremediation (Crawford and Mohn 1985; Lamar and Evans 1993; Miethling and Karlson 1996). The position of the chlorine substitution and the number of chlorines influence how the chlorophenols are degraded by microorganisms. Because of the presence of six isomers of trichlorophenols, three isomers of tetrachlorophenols and one pentachlorophenol, various microorganisms have evolved different strategies for the degradation of selected isomers. Bacteria can degrade polychlorophenols under both aerobic and anaerobic conditions, and fungi are able to aerobically metabolize them.

### *1.2.1 Pentachlorophenol Degradation by Aerobic Bacteria*

Chu and Kirsch (1972) reported the first aerobic PCP-degrading bacterium in 1972. Since then, numerous aerobic bacteria that degrade PCP have been isolated from different regions around the globe. The early isolates were originally assigned to various genera, such as *Arthrobacter*, *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. The gram-positive *Rhodococcus* spp. and *Mycobacterium* spp. have been reclassified as *Mycobacterium chlorophenolicum* (Briglia et al. 1994; Haggblom et al. 1994). All the gram-negative, PCP-degrading bacteria, previously known as *Arthrobacter*, *Pseudomonas*, and *Flavobacterium*, were subsequently reclassified as *Sphingomonas chlorophenolica* strains (Crawford and Ederer 1999; Takeuchi et al. 2001), but have been subsequently renamed as *Sphingobium chlorophenolicum* strains (Takeuchi et al. 2001). A PCP-degrading *Sphingomonas* sp. strain UG30A is related to *S. chlorophenolicum* strains, but remains as a *Sphingomonas* sp. (Habash et al. 2009). A related psychrophilic PCP-degrader is *Novosphingobium lentum* MT1 (Tiirola et al. 2005). *S. chlorophenolicum* strains are the most frequently isolated bacteria that degrade PCP; however, other PCP-degrading bacteria have also been reported (Golovleva et al. 1992; Sharma et al. 2009).

### 1.2.2 2,4,6-Trichlorophenol Degradation by Aerobic Bacteria

Although *S. chlorophenolicum* degrades both PCP and 2,4,6-TCP (Steiert et al. 1987), *Azotobacter* sp. GP1 (Li et al. 1991) and *Ralstonia* (ex. *Pseudomonas*) *pickettii* (Kiyohara et al. 1992) use only 2,4,6-TCP as a sole carbon source. More 2,4,6-TCP degraders have since been identified and isolated: *Cupriavidus necator* (ex. *Ralstonia eutrapha*) JMP134 (Clement et al. 1995), *Sphingopyxis chilensis* (ex. *Pseudomonas paucimobilis*) S37 (Aranda et al. 1999), *Aureobacterium* sp. C964 (Bock et al. 1996), *Rhodococcus percolatus* MBS1T (Briglia et al. 1996), *Sphingobium subarctica* (Puhakka et al. 1995; Nohynek et al. 1996), *Pseudomonas* sp., *Agrobacterium* sp. (Wang et al. 2000), *Nocardioides* sp. (Mannisto et al. 1999), *Flavobacterium* sp. and *Caulobacter* sp. (Mannisto et al. 1999). It appears that the 2,4,6-TCP degrading ability is widespread among the soil bacteria.

### 1.2.3 2,4,5-Trichlorophenol Degradation by Aerobic Bacteria

Several bacteria are known to degrade 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). *Burkholderia* (ex *Pseudomonas*) *cepacia* AC1100, isolated from an enrichment culture, is a gram-negative bacterium that uses 2,4,5-T as a sole carbon source for the growth (Kilbane et al. 1982). The bacterium degrades 2,4,5-T with 2,4,5-TCP as the first metabolic intermediate (Karns et al. 1983). Two other *Burkholderia* spp. that degrade 2,4,5-T have recently been reported (Lü et al. 2003; Rice et al. 2005). A different 2,4,5-T degrader is *Nocardioides simplex* 3E that is a gram-positive actinomycete, able to grow on 2,4,5-T as a sole carbon source (Golovleva et al. 1990). This microorganism may have two pathways for 2,4,5-T degradation: one with 2,4,5-TCP as the first metabolic intermediate, and the other with dichlorohydroxyphenoxyacetate as the first metabolic intermediate. Since 2,4,5-TCP degradation is an integral part of 2,4,5-T degradation, the characterized pathway for 2,4,5-T degradation is reviewed here.

### 1.2.4 Anaerobic Degradation of Polychlorophenols

Microorganisms also degrade polychlorophenols under anaerobic conditions. Reductive dechlorination of PCP to tetrachlorophenols, trichlorophenols, dichlorophenols, and monochlorophenols was first observed in anaerobic paddy soils in the 1970s (Ide et al. 1972). The degradation has been confirmed by studies with enrichment cultures and bacterial isolates. An anaerobic bacterial consortium completely dechlorinates PCP to phenol and then mineralizes the produced phenol (Mikesell and Boyd 1986). *Desulfitobacterium frappieri* converts PCP by sequential reductive dehalogenation to 3-chlorophenol (Bouchard et al. 1996). These anaerobic bacteria use polychlorophenols as terminal electron acceptors for anaerobic

respiration to produce less substituted chlorophenols and phenol (Crawford and Mohn 1985), and these phenols are further degraded by other organisms in enrichment cultures or in the environment (Mikesell and Boyd 1986). Progress has been made towards understanding the biochemistry and genetics of reductive dechlorination of polychlorophenols (Boyer et al. 2003; Bisaillon et al. 2010).

### *1.2.5 Fungal Degradation of Polychlorophenols*

Fungal degradation of PCP was reported as early as 1960s (Duncan and Deverall 1964), and the non-specific breakdown of PCP by fungal laccase, tyrosinase, and peroxidase was implied (Lyr 1963). Research on fungal degradation of polychlorophenols has progressed rapidly since then, especially with white-rot fungi (Reddy et al. 1998; Reddy and Gold 2000). The metabolic pathways of 2,4,6-TCP and PCP degradation have been studied with cell extracts of white-rot fungus *Phanerochaete chrysosporium* (Reddy et al. 1998; Reddy and Gold 2000), and a glutathione conjugate reductase involved in PCP degradation has been purified and characterized (Reddy and Gold 2001). *Phanerochaete* spp. have been used for the removal of PCP from contaminated soils (Lamar and Dietrich 1990; Lamar and Evans 1993) and for the disposal of PCP-treated woods (Lamar and Dietrich 1992).

## 1.3 Biochemistry of Polychlorophenol Degradation

The aerobic breakdown of aromatic compounds starts with monooxygenases or dioxygenases that introduce hydroxyl groups into the aromatic rings. Many aromatic compounds, including phenol, benzene and anthranilate, are converted to catechol or substituted catechols. Then intradiol or extradiol catechol dioxygenases break the aromatic rings to produce aliphatic compounds, which are further channelized into the tricarboxylic acid cycle for the complete mineralization (Harwood and Parales 1996). However, polychlorinated phenols are converted to substituted quinols before ring-cleavage: *S. chlorophenolicum* L-1 (ex. *S. chlorophenolicum* ATCC 39723) metabolizes PCP to 2,6-dichloroquinol (Cai and Xun 2002), *C. necator* JMP134 converts 2,4,6-TCP to 6-chlorohydroxyquinol (Louie et al. 2002), and *B. cepacia* AC1100 channels 2,4,5-TCP to hydroxyquinol (Zaborina et al. 1998).

### *1.3.1 Pentachlorophenol Metabolic Pathway of S. Chlorophenolicum L-1*

PCP degradation pathways have been thoroughly investigated in *S. chlorophenolicum* L-1 and partially studied in *Mycobacterium chlorophenolicum*. Studies with cell

extracts of *M. chlorophenolicum* have shown that PCP is converted to tetrachloroquinol and then to hydroxyquinol before ring-cleavage (Apajalahti and Salkinoja-Salonen 1987; Uotila et al. 1995). The details of this pathway are unknown, as the enzymes and genes have not been identified. For PCP degradation by *S. chlorophenolicum* L-1, the complete degradation pathway has been determined, which is different from that of *M. chlorophenolicum* (Cai and Xun 2002). *S. chlorophenolicum* L-1 is isolated from a PCP-contaminated soil and it is able to completely mineralize PCP to $CO_2$ and chlorine (Saber and Crawford 1985). Genetic approaches were initially used to study the metabolic pathway in *S. chlorophenolicum* L-1; however, transposon mutagenesis has been unsuccessful (Orser and Lange 1994) and chemical mutagenesis has produced a single mutant (Steiert and Crawford 1986). Since a plasmid vector that replicates in *S. chlorophenolicum* L-1 has not been obtained, the mutated gene cannot be identified by complementation.

#### 1.3.1.1 Pentachlorophenol 4-Monooxygenase (PcpB)

The metabolic pathway has been elucidated from biochemical studies and confirmed by gene inactivation (Fig. 1.1). In order to characterize PCP metabolizing enzyme, a convenient assay has to be developed. Detection of chlorine released from PCP metabolism would be a logical choice, but most buffers have relatively high background levels of chlorine that interferes with the assay. Since PCP-induced *S. chlorophenolicum* L-1 cells also degrade 2,4,6-triiodophenol, an enzyme that release iodide from 2,4,6-triiodophenol is identified in the cell extracts of PCP-induced *S. chlorophenolicum* L-1 (Xun and Orser 1991c) and purified (Xun and Orser 1991a). The protein (PcpB) is a flavin protein, containing a single flavin adenine dinucleotide (FAD). PcpB converts not only 2,4,6-triiodophenol to 2,6-diiodoquinone but also PCP to tetrachloroquinone with the consumption of NADH and $O_2$. 2,6-diiodoquinone and tetrachloroquinone are rapidly reduced to 2,6-diiodoquinol and tetrachloroquinol by NADH or ascorbic acid added in the reaction mixture (Xun et al. 1992c; Dai et al. 2003; Belchik and Xun 2008). The gene (*pcpB*) is cloned by using a probe of degenerated oligonucleotides designed according to the N-terminal sequence of PcpB (Orser et al. 1993a). When *pcpB* is inactivated by homologous recombination, the *pcpB* mutant is unable to degrade PCP (Lange et al. 1996; Cai and Xun 2002). PcpB has a calculated molecular weight of 59,932, and it is homologous to other FAD-dependent monooxygenases that hydroxylate aromatic rings. PcpB has a broad substrate range, hydroxylating the *para* position of several substituted phenols, including PCP, 2,3,5,6-tetrachlorophenol, 2,4,6-triiodophenol, 2,4,6-tribromophenol, 2,4,6-TCP, and 2,6-dichlorophenol (Xun et al. 1992c). As long as the 2,6-positions are occupied by chlorine, bromine, or iodine, PcpB can hydroxylate the *para*-position of the substituted phenols. When the *para*-position is substituted, the substituted group will be removed after PcpB catalysis. The reaction consumes one NADH when the substituted group is an electron-donating group, such as a

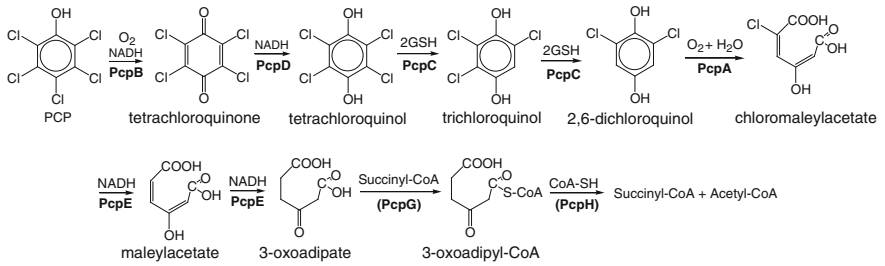


**Fig. 1.1** PCP degradation pathway of *S. chlorophenolicum* L-1. PcpB, PCP 4-monooxygenase; PcpD, tetrachloroquinone reductase; PcpC, tetrachloroquinol reductive dehalogenase; PcpA, 2,6-dichloroquinol 1,2-dioxygenase; PcpE, chloromaleylacetate reductase; PcpG, 3-oxoadipate: succinyl-CoA transferase; and PcpH, 3-oxoadipyl-CoA thiolase. PcpG and PcpH are hypothetical, but they are common enzymes in biodegradation of aromatic compounds

hydrogen or an amino group. On the other hand, the overall reaction consumes two NADH when the substituted group is an electron-withdrawing group, i.e. halogen, nitro group, or cyano group.

### 1.3.1.2 Tetrachloroquinone Reductase (PcpD)

The consumption of two NADH for the dehalogenation of substituted phenols is carefully examined with *p*-hydroxybenzoate 3-monooxygenase, a related FAD-dependent monooxygenase (Husain et al. 1980). For fluoride elimination from tetraflouro-*p*-hydroxybenzoate, the direct product is a quinone, which is chemically reduced by NADH to produce triflouro-3,4-dihydroxybenzoate, and the overall reaction consumes two NADH. *p*-Hydroxybenzoate 3-monooxygenase has been extensively studied as a model system for flavin containing monooxygenases. The normal hydroxylation reaction produces a non-aromatic compound as a transient reaction intermediate, which rearranges back to an aromatic compound (Entsch et al. 1976). For halogen removal, a more oxidized non-aromatic intermediate is generated, and it rearranges to produce a quinone (Husain et al. 1980). The authors have suggested that halide and nitrite elimination catalyzed by similar monooxygenases should also produce quinones. This prediction has been validated and expanded. First, Haigler et al. (1996) have reported that a *Burkholderia* DNT4-methyl-5-nitrocatechol 5-monooxygenase converts 4-methyl-5-nitrocatechol to 2-hydroxy-5-methylquinone with nitrite elimination, and a quinone reductase reduces the quinone to 2-hydroxy-5-methylquinol. Second, the gene immediately after *pcpB* is *pcpD*, whose translated product is homologous to the reductase component of several oxygenases (Orser and Lange 1994). The function of PcpD had remained elusive for several years, until Dai et al. (2003) reported that PcpD reduces tetrachloroquinone with NADH as the reductant (Fig. 1.1). PcpD is not required for PCP 4-monooxygenase to convert PCP to tetrachloroquinone, which can be reduced either

enzymatically by PcpD or chemically by reducing agents. However, PcpD enhances PCP 4-monooxygenase activity under in vitro conditions. A *pcpD* mutant can degrade PCP at a reduced rate, and the mutant is more sensitive to high concentrations of PCP. Thus, PcpD is named as tetrachloroquinone reductase.

#### 1.3.1.3 Tetrachloroquinol Reductive Dehalogenase (PcpC)

The third and fourth steps of PCP degradation (Fig. 1.1) are catalyzed by PcpC, tetrachloroquinol reductive dehalogenase, with glutathione (GSH) as the reducing agent. Tetrachloroquinol is expected to be converted to 2,6-dichloroquinol because a mutant obtained by chemical mutagenesis accumulates the latter (Steiert and Crawford 1986), and the reaction is believed to be reductive dechlorination. The reductive conversion of tetrachloroquinol to 2,6-dichloroquinol is observed with the cell extracts under anaerobic conditions, which are essential for the initial observation, as the reaction is slow and tetrachloroquinol is unstable in the presence of oxygen. When several known cellular reducing agents are tested as the reducing power, only GSH significantly stimulates the reaction rate (Xun et al. 1992b). Subsequently, tetrachloroquinol reductive dehalogenase (PcpC), a glutathione transferase, is purified from the cell extracts of *S. chlorophenolicum* L-1 (Xun et al. 1992a). The enzyme converts tetrachloroquinol to trichloroquinol and then 2,6-dichloroquinol with the concomitant oxidation of GSH. The corresponding gene (*pcpC*) has been cloned by using probes designed from the N-terminal sequence of the purified protein, and the sequence determined (Orser et al. 1993b). PcpC is necessary for *S. chlorophenolicum* L-1 to completely degrade PCP, as a *pcpC* mutant accumulates tetrachloroquinol during PCP degradation. Tetrachloroquinol is unstable in the presence of oxygen and is further transformed into a colored compound and accumulated in the culture medium (Cai and Xun 2002). PcpC is slightly homologous to the Zeta class of glutathione transferases that catalyze maleylacetoacetate isomerization, a reaction in tyrosine metabolism. Despite low sequence similarity (19.4% sequence identity with human GSTZ1), PcpC has maleylacetoacetate isomerase activity and is characterized as a Zeta-class glutathione transferase (Anandarajah et al. 2000). However, a comprehensive phylogenetic analysis groups PcpC with LinD, another chloroquinol reductive dehalogenase and the analysis separate them from the Zeta class of glutathione transferases (Marco et al. 2004).

#### 1.3.1.4 2,6-Dichloroquinol 1,2-Dioxygenase (PcpA)

The third essential enzyme in the PCP degradation pathway is PcpA, which is the first identified protein involved in PCP degradation. When *S. chlorophenolicum* L-1 cells are treated with EDTA, a chelating agent, the cells lose their ability to degrade PCP and several major proteins are released to the solution (Xun and Orser 1991b). One of the EDTA released proteins is present in the PCP-induced

cells, but absent from the uninduced cells. The protein is purified, and the corresponding gene is cloned; however, the gene product had no homology to any entries in the GenBank in 1991. Thus, PcpA was reported as a PCP-induced protein with unknown function in 1991 (Xun and Orser 1991b). In 1999, three groups reported PcpA as a ring-cleavage dioxygenase (Ohtsubo et al. 1999; Xu et al. 1999; Xun et al. 1999). PcpA is 2,6-dichloroquinol 1,2-dioxygenase that oxidizes 2,6-dichloroquinol to 2-chloromaleylacetate chloride (Fig. 1.1), which is spontaneously hydrolyzed to 2-chloromaleylacetate (Fig. 1.1) (Ohtsubo et al. 1999; Xun et al. 1999). PcpA has a molecular weight of 36,513 and requires free $Fe^{2+}$ as a cofactor. PcpA was originally thought to be a periplasmic protein because of its release from EDTA treatment of *S. chlorophenolicum* L-1 cells (Xun and Orser 1991b). Given the requirement of free $Fe^{2+}$ as a cofactor ($K_m$ value of 5 μM $Fe^{2+}$) (Xun et al. 1999), which is available only in the cytoplasm of aerobic bacteria (Raymond et al. 2003), PcpA should be a cytoplasmic protein, the same as other enzymes of the PCP degradation pathway, all of which require cofactors available in the cytoplasm. PcpA is required for PCP metabolism because a *pcpA* inactivation mutant accumulates 2,6-dichloroquinol (Chanama and Crawford 1997). PcpA has 51.6% of amino acid sequence identity to LinE, which is a 2-chloroquinol 1,2-dioxygenase (Ohtsubo et al. 1999), essentially catalyzing the same reaction as PcpA. LinE also oxidizes quinol to gamma-hydroxymuconic semialdehyde. Other hypothetical proteins, e.g. YdfO (PIR:E69781), YodE (PIR:B69903) and YkcA (PIR:C69855) of *Bacillus subtilis*, similar to PcpA and LinE, are likely quinol ring-cleavage dioxygenases. A recent BLAST search of microbial genomes with PcpA (October 5, 2010) identified many PcpA homologs (more than 30% sequence identity), and they are quite common in Halobacteria of Archaea, Gram-positive bacteria (*Bacillus* and *Lactorbacillus*) and Gram-negative bacteria (alpha Proteobacteria). *S. chlorophenolicum* is an alpha Proteobacterium.

#### 1.3.1.5 2-Chloromaleylacetate Reductase (PcpE)

2-Chloromaleylacetate is a relatively common metabolic intermediate for the degradation of chloroaromatic compounds, and it is reduced by (chloro)maleylacetate reductase to maleylacetate and then 3-oxoadipate with the consumption of NADH (Kaschabek et al. 2002). In *S. chlorophenolicum* L-1, a (chloro) maleylacetate reductase reduces 2-chloromaleylacetate to 3-oxoadipate (Cai and Xun 2002) (Fig. 1.1). The reductase gene (*pcpE*) is identified by sequencing DNA around *pcpA*. PcpE is involved in PCP degradation in *S. chlorophenolicum* L-1 because a *pcpE* inactivation mutant transitorily accumulates 2-chloromaleylacetate during PCP degradation. The result also suggests that *S. chlorophenolicum* L-1 has additional maleylacetate reductase that is produced when 2-chloromaleylacetate is accumulated in the mutant culture. A BLAST search of the recently released *S. chlorophenolicum* L-1 genome identified two additional genes coding for maleylacetate reductases.

#### 1.3.1.6 3-Oxoadipate:succinyl-CoA Transferase and 3-Oxoadipyl-CoA Thiolase

3-Oxoadipate is a common metabolic intermediate of many aromatic compounds, and two additional enzymes are required to channel it into the tricarboxylic acid cycle for complete mineralization (Harwood and Parales 1996). The enzymes have been characterized in other bacteria, but not in *S. chlorophenolicum* (Kaschabek et al. 2002). The first enzyme is 3-oxoadipate:succinyl-CoA transferase that catalyzes the CoA exchange between 3-oxoadipate and succinyl-CoA to generate 3-oxoadipyl-CoA and succinate. The enzyme consists of subunits A and B. The *S. chlorophenolicum* L-1 genome has two sets of genes potentially coding for oxoadipate:succinyl-CoA transferases. One set of the genes is located next to a gene encoding 3-oxoadipyl-CoA thiolase that catalyzes the conversion of 3-oxoadipyl-CoA and CoA-SH to succinyl-CoA and acetyl-CoA. Both succinyl-CoA and acetyl-CoA are completely mineralized via the tricarboxylic acid cycle.

#### 1.3.1.7 Glutathionyl-(chloro)quinol Reductase (PcpF)

Glutathionyl-(chloro)quinol reductase (PcpF) plays a maintenance role of the PCP metabolic pathway. PcpC catalyzes the GSH-dependent conversion of tetrachloroquinol to trichloroquinol and then to 2,6-dichloroquinol (Xun et al. 1992a; Orser et al. 1993b). The Cys-14 residue of PcpC is required for the reaction (Warner et al. 2005). When the Cys-14 residue is oxidized (or mutated), the oxidatively-damaged PcpC (PcpC-ox) transforms tetrachloroquinol to glutathionyl-trichloroquinol (GS-trichloroquinol) and trichloroquinol to GS-dichloroquinol (Fig. 1.2). PcpC and PcpC-ox cannot further transform GS-trichloroquinol and GS-dichloroquinol. Another enzyme, PcpF, catalyzes GSH-dependent reduction of GS-trichloroquinol and GS-dichloroquinol to trichloroquinol and dichloroquinol, respectively, which re-enter the PCP degradation pathway (Fig. 1.2) (Huang et al. 2008). PcpF plays a maintenance role but not essential for PCP degradation by *S. chlorophenolicum* L-1. When the *pcpF* gene is inactivated, the mutant degrades PCP more slowly and becomes more sensitive to PCP toxicity than the wild type.

PcpF is discovered due to its gene location next to the *pcpC* gene on the chromosome of *S. chlorophenolicum* L-1. Both PcpC and PcpF are glutathione transferases. The two proteins share 17.7% sequence identity. PcpC does not share high sequence identity with any glutathione transferase in sequenced bacterial genomes. The closest relative with 27.3% sequence identity is a hypothetical glutathione transferase (GenBank: AAZ25344) of *Colwellia psychrerythraea* 34H. PcpF is completely different. It has homologs (more than 30% sequence identity) in halobacteria (Archaea), bacteria (Bacteria), fungi (Eukarya), and plants (Eukarya). *S. cerevisiae* has three PcpF homologs (YGR154c, ECM4, YMR251w) with ECM4 (extra cellular mutant 4) being the most similar to PcpF. Although they were initially characterized as Omega-class glutathione transferases for their ability to use the substrates of the Omega-class glutathione transferases (Garcera

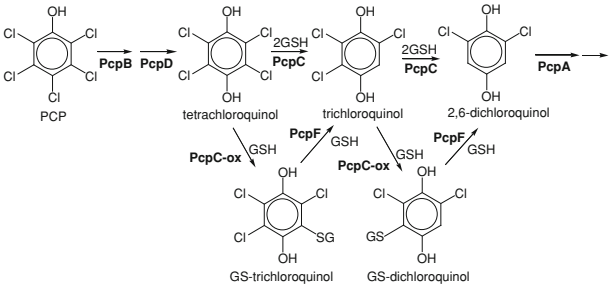


**Fig. 1.2** The maintenance role of PcpF in PCP degradation by *S. chlorophenolicum* L-1. PcpC-ox oxidatively damaged PcpC; PcpF, GS-(chloro)quinol reductase

et al. 2006), they share less than 20% sequence identity with the Omega-class glutathione transferases. *A. thaliana* has four PcpF homologs, listed as ECM4-like proteins (NP_199315, NP_001031671, NP_199312, NP_568632).

#### 1.3.1.8 PcpF Homologs are GS-(chloro)quinol Reductases

Phylogenetic analsysis grouped PcpF homologs into a distinct group, separated from any glutathione transferase class. Several bacterial PcpF homolog and yeast ECM4 all actively reduced GS-trichloroquinol. Thus, they are characterized as GS-(chloro)quinol reductases, a new class of glutathione transferases (Xun et al. 2010). GS-(chloro)quinol reductases can also reduce GS-quinol to quinol at the expense of GSH (Lam and Xun, unpublished data). They are related to the newly discovered Omega class, Lambda class and dehydroascorbate (DHA) reductases of glutathione transferases. Unlike other glutathione transferases, the new classes have little or no activity for transferring GSH to electrophilic compounds; however, they catalyze GSH-dependent reductions. The Lambda-class glutathione transferases perform only thiol transfer, and DHA reductases catalyze both thiol transfer and the reduction of DHA to ascorbate (Dixon et al. 2002). The Omega-class glutathione transferases catalyze GSH-dependent reductions of disulfide bonds (thiol transfer), DHA, and dimethylarsinate (Whitbread et al. 2005). Glutathionyl-(chloro)hydroquinone reductases not only catalyze the activities of the Omega-class glutathione transferases, but also reduce GS-trichloroquinol to trichloroquinol (Xun et al. 2010).

*S. chlorophenolicum* L-1 uses the PCP-metabolizing enzymes for the degradation of other polychlorophenols. *S. chlorophenolicum* L-1 is also able to mineralize a broad spectrum of chlorinated phenols, as long as the 2,6-positions are substituted with chlorines (Steiert et al. 1987). The roles of PcpB, PcpC, and PcpA in degrading these compounds are summarized in Fig. 1.3. It now becomes clear why the 2 and 6-substitutions are required for the cells to completely degrade the chlorinated phenols, as 2,6-dichloroquinol is the necessary metabolic intermediate before PcpA can break the aromatic ring. Besides chlorinated phenols, bromophenols and iodophenols are degraded by the same enzymes (Xun and

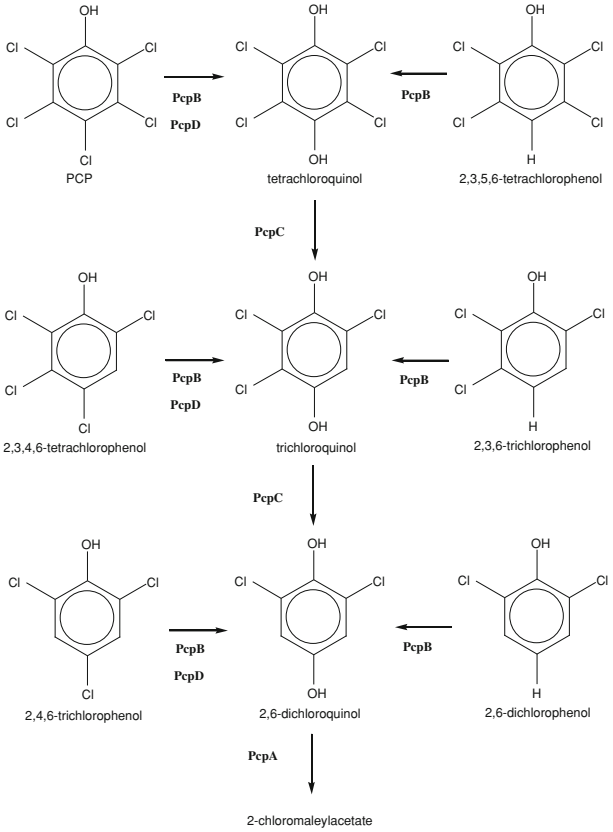


**Fig. 1.3** The versatility of the PCP-degrading enzymes for the degradation of polychlorophenols. *S. chlorophenolicum* L-1 degrades several polychlorophenols as long as the 2,6-positions are occupied with chlorines. The combined actions of PcpB, PcpD, PcpC, and PcpA are responsible for the degradation. When PcpB dechlorinates, a quinone is formed. PcpD can reduce the quinone to the corresponding quinol

Orser 1991c; Xun et al. 1992c). Further, most of the halogenated phenols can induce the expression of the PCP degrading genes (Cai and Xun 2002). The *pcpB*, *pcpC*, and *pcpA* homologues have been identified from other PCP degrading *S. chlorophenolicum* strains and *Novosphingobium* spp. (Ederer et al. 1997; Tiirola et al. 2002). Thus, it is reasonable to conclude that the sphingomonads may use homologous enzymes for polychlorophenol degradation.

### *1.3.2 2,4,6-Trichlorophenol Metabolic Pathway of C. Necator JMP134*

*C. necator* JMP134, best known for its ability to degrade 2,4-dichlorophenoxyacetate, also degrades 2,4,6-TCP (Clement et al. 1995). The initial proposed pathway for

2,4,6-TCP degradation includes 2,6-dichloroquinol as a metabolic intermediate (Padilla et al. 2000). The enzymes responsible for 2,4,6-TCP degradation in *C. necator* JMP134 have been identified, purified, and characterized by our group (Louie et al. 2002). Further characterization of 2,4,6-TCP 4-monooygenase has shown that 2,6-dichloroquinol is a by-product of the enzyme and not a metabolic intermediate in *C. necator* JMP134 (Xun and Webster 2004).

#### 1.3.2.1 The *tcp* Gene Cluster

When *C. necator* JMP134 grows on sodium glutamate, it does not degrade 2,4,6-TCP. After glutamate is used up, JMP134 rapidly consumes 2,4,6-TCP, indicating the expression of 2,4,6-TCP degrading genes is subject to catabolic repression (Louie et al. 2002). 2,4,6-TCP 4-monooxygenase (TcpA) is partially purified from JMP134 cell extracts and characterized as a reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase. The corresponding gene is identified by PCR using primers designed from conserved regions of $FADH_2$-dependent monooxygenases, and a gene cluster (*tcpABC*) has been determined. *tcpA* encodes the $FADH_2$-dependent 2,4,6-TCP 4-monooxygenase, and *tcpC* codes for 6-chlorohydroxyquinol 1,2-dioxygenase, respectively. The three genes have been individually inactivated in *C. necator* JMP134. The *tcpA* mutant fails to degrade 2,4,6-TCP; the *tcpB* mutant is not essential for 2,4,6-TCP degradation; and the *tcpC* mutant accumulated 6-chlorohydroxyquinol from 2,4,6-TCP degradation (Louie et al. 2002). After the genome of *C. necator* JMP134 is sequenced, a complete gene cluster *tcpRXABCYD* involved in TCP degradation is identified (Matus et al. 2003). The *tcpR* gene codes for a gene regulator that is required for the expression of the other *tcp* genes (Sánchez and González 2007). The functions of these *tcp* gene products have been characterized with recombinant proteins produced in *Escherichia coli*.

#### 1.3.2.2 2,4,6-Trichlorophenol 4-Monooxygenase (TcpA)

Recombinant TcpA, purified from *E. coli*, transforms 2,4,6-TCP to 6-chlorohydroxyquinol when $FADH_2$ is supplied by an NADH:FAD oxidoreductase of *E. coli* (Louie et al. 2002). 2,6-dichloroquinol is accumulated to a marginal amount in the reaction mixture, but is hardly consumed by TcpA, questioning whether 2,6-dichloroquinol is a real metabolic intermediate. Further investigation has demonstrated that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol according to the scheme presented in Fig. 1.4 (Xun and Webster 2004). Because dechlorination of phenolic compounds by monooxygenases produces quinones (Husain et al. 1980; Haigler et al. 1996; Dai et al. 2003), TcpA oxidizes 2,4,6-TCP to 2,6-dichloroquinone. TcpA then uses the product, while it is still bound to the active site, as its second substrate and removes the second chlorine by hydrolysis to generate 6-chlorohydroxyquinone, which is chemically or enzymatically reduced to

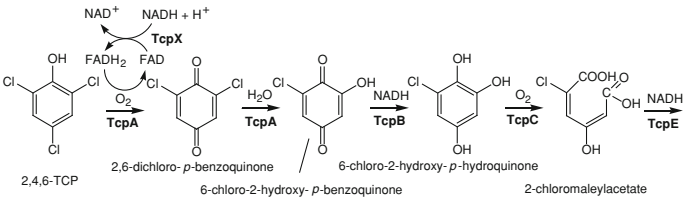


**Fig. 1.4** 2,4,6-TCP degradation pathway of *C. necator* JMP134. TcpA, 2,4,6-TCP 4-monooxygenase; TcpB, quinone reductase; TcpC, 6-chlorohydroxyquinol 1,2-dioxygenase; and TcpD, chloromaleylacetate reductase

6-chlorohydroxyquinol. When TcpA oxidizes 2,6-dichlorophenol, it simply introduces a hydroxyl group at the *para*-position and produces 2,6-dichloroquinol, which is not further metabolized by TcpA.

TcpA belongs to $FADH_2$-dependent monooxygenases. *E. coli* 4-hydroxyphenylacetate 3-monooxygenase (Galán et al. 2000; Xun and Sandvik 2000), TcpA (Louie et al. 2002), chlorophenol 4-monooxygenase (TftD) (Gisi and Xun 2003), and phenol 2-monooxygenase (Kirchner et al. 2003) are the early reported $FADH_2$-dependent monooxygenases. Sequence analysis suggests that a group of aromatic compound-hydroxylating enzymes are $FADH_2$-dependent monooxygenases, including 2,4,6-TCP 4-hydroxylase of *Ralstonia pickettii* DTP0602 (Takizawa et al. 1995), PhzO for biosynthesis of 2-hydroxylated phenazine antibiotics in *Pseudomonas aureofaciens* 30-84 (Delaney et al. 2001), PvcC involved in siderophore synthesis (Stintzi et al. 1996), and many hypothetical enzymes from GenBank. The characterized $FADH_2$-dependent monooxygenases are involved in biodegradation of aromatic compounds and biosynthesis of antibiotics and siderophores. Since TcpA does not have additional domains in comparison to other $FADH_2$-dependent monooxygenases, it is different from bifunctional enzymes that have two functional domains. Therefore, its ability to catalyze the hydrolytic reaction must be due to catalytic promiscuity, describing the ability of certain enzymes to catalyze unrelated reactions with the same active site.

#### 1.3.2.3 NADH:FAD Oxidoreductase (TcpX)

The *tcpX* gene is immediately upstream *tcpA* in the *tcp* gene cluster, *tcpRXABCYD*. Since TcpX is highly homologous to the characterized flavin reductases (TftC and HpaC) that supply $FADH_2$ to $FADH_2$-dependent monooxygenases, TcpX is expected to be the flavin reductase that provides TcpA with $FADH_2$ (Matus et al. 2003). The recombinant TcpX produced in *E. coli* actively reduces FAD or FMN with NADH as the reducing power (Belchik and Xun 2008). Because $FADH_2$ is unstable in the presence of $O_2$, it is generated by TcpX in the reaction mixture and used by TcpA to oxidize 2,4,6-TCP (Fig. 1.4). TcpA can also

use $FADH_2$ generated by other flavin reductases. In fact, TcpA is characterized with a general *E. coli* flavin reductase (Fre) that provides $FADH_2$ for TcpA (Louie et al. 2002; Xun and Webster 2004).

#### 1.3.2.4 Quinone Reductase (TcpB)

TcpB belongs to the nitroreductase family (pfam00881) (Bateman et al. 2002) of flavin reductases. The enzymes in this family catalyze the reduction of nitroaromatics, quinones, and flavins. Although some family members, e.g. *Vibrio harveyi* NADPH:FMN oxidoreductase and *V. fischeri* NAD(P)H:FMN oxidoreductase, provide bacterial luciferases with $FMNH_2$ (Inouye 1994; Lei et al. 1994), TcpB does not reduce FAD. TcpB is a quinone reductase with menadione as the best substrate among several tested substrates, including FAD, FMN, and Ferricyanide (Belchik and Xun 2008). The reduction of 6-chlorohydroxyquinone to 6-chlorohydroxyquinol by TcpB is indirectly confirmed as TcpB stimulates TcpA activity for 2,4,6-TCP oxidation in vitro. Although 6-chlorohydroxyquinone can be chemically reduced by reducing agents, such as NADH and ascorbic acid, TcpB is more efficient to reduce 6-chlorohydroxyquinone. 6-Chlorohydroxyquinone spontaneously reacts with thiols to form conjugates, and TcpB minimizes the formation of the conjugates. Thus, TcpB effectively reduces 6-chlorohydroxyquinone to 6-chlorohydroxyquinol and protects cellular thiols.

#### 1.3.2.5 6-Chlorohydroxyquinol 1,2-Dioxygenase (TcpC)

TcpC oxidizes 6-chlorohydroxyquinol to 2-chloromaleylacetate (Louie et al. 2002). It is a member of the intradiol ring-cleavage dioxygenases, consisting of all the characterized (chloro)hydroxyquinol dioxygenases together with some catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases. Since inactivation of the *tcpC* also causes the mutant to accumulate 6-chlorohydroxyquinol, the data collectively support that JMP134 transforms 2,4,6-TCP to 2-chloromaleylacetate by TcpA and TcpC (Fig. 1.4). In addition, TcpX, a flavin reductase, is required to generate $FADH_2$ for TcpA, and TcpB reduces 6-chlorohydroxyquinone to 6-chlorohydroxyquinol.

#### 1.3.2.6 Chloromaleylacetate Reductase (TcpD)

TcpD and PcpE share 52.9% sequence identity, suggesting that TcpD is a chloromaleylacetate reductase. When the *tcpD* gene is inactivated, the mutant lost the ability to grow with 1.5 mM 2,4,6-TCP as the sole carbon and energy source (Sánchez and González 2007). Although TcpD is not characterized, it is expected to catalyze the same reactions as PcpE (Fig. 1.1) to convert 2-chloromaleylacetate to maleylacetate and then to 3-oxoadipate (Fig. 1.4).

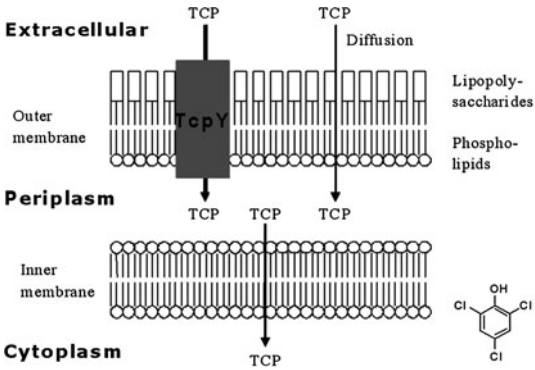


**Fig. 1.5** Proposed entry of 2,4,6-TCP via diffusion and through TcpY into *C. necator* JMP134. TCP diffuses through cytoplasmic membranes, but the diffusion may be slowed by lippolysaccharides of outer membrane. TcpY may serve as a TCP channel

### 1.3.2.7 *β*-Barrel Outer Membrane Protein (TcpY)

TcpY facilitates the uptake of 2,4,6-trichlrophenol into *C. necator* (Belchik et al. 2010). Except *tcpY*, the inactivation of other genes in the *tcpRXABCYD* gene cluster negatively affects 2,4,6-TCP degradation by *C. necator* JMP134. Inactivation of *tcpY* has no apparent effect on 2,4,6-TCP degradation. Sequence analysis associates TcpY with COG4313, a group of hypothetical proteins. Signal peptide, structure and topology analyses indicate that TcpY is a *β*-barrel outer membrane protein of Gram negative bacteria. Structurally similar proteins include *Escherichia coli* outer membrane protein FadL that transports hydrophobic, long-chain fatty acids across the hydrophilic outer leaflet of the outer membrane, consisting of lipopolysaccharides. Several lines of evidence support that TcpY facilitates the uptake of 2,4,6-TCP into *C. necator*. First, constitutive expression of *tcpY* in two *C. necator* strains rendered the cells more sensitive to polychlorophenols, including 2,4,6-TCP. Second, *C. necator* JMP134 expressing cloned *tcpY,* transported more 2,4,6-TCP into the cell than it carrying the cloning vector. 2,4,6-TCP may enter the cell by diffusion or through other *β*-barrel outer membrane proteins (Fig. 1.5). We believe that 2,4,6-TCP uptake is not the limiting step for its degradation by *C. necator* JMP134.

### 1.3.2.8 The Presence of *tcp* Genes in Other Bacteria

2,4,6-TCP degradation pathways have also been studied in other 2,4,6-TCP degraders that do not mineralize PCP. Chlorohydroxyquinol 1,2-dioxygenases have been identified, purified, and characterized from 2,4,6-TCP-degrading *Azotobacter* sp. GP1 (Zaborina et al. 1995) and *Streptomyces rochei* 303 (Zaborina et al. 1995), and the enzymatic activities have been observed from 2,4,6-TCP degrading *Ralstonia pickettii* (Takizawa et al. 1995). 2,4,6-TCP 4-monooxygenases have been reported from *R. pickettii* (Takizawa et al. 1995) and *Azotobacter* sp. GP1 (Wieser et al. 1997). The two 2,4,6-TCP 4-monooxygenases have high

sequence identity with TcpA (Louie et al. 2002). The *Azotobacter* enzyme has been highly purified and assayed without providing $FADH_2$ as a co-substrate, and the reported enzyme activity (Wieser et al. 1997), which is 47 folds lower than that of TcpA (Louie et al. 2002), is likely due to the insufficient supply of $FADH_2$ in the reaction mixture. Perhaps, due to the low measurable enzyme activity, the main end product 6-chlorohydroxyquinol is not identified from the oxidation of 2,4,6-TCP by the enzyme (Wieser et al. 1997). Thus, the metabolic pathway from earlier studies is incomplete as the link from 2,4,6-TCP to 2-chlorohydroxyquinol is missing, but the studies have laid the foundation for the complete characterization of 2,4,6-TCP degradation pathway in *C. necator* JMP134 (Louie et al. 2002). Since these 2,4,6-TCP degraders all have homologous 2,4,6-TCP 4-monooxygenases and chlorohydroxyquinol 1,2-dioxygenases (Louie et al. 2002; Matus et al. 2003), they should have the same pathway for 2,4,6-TCP degradation (Fig. 1.4). Further, because *S. chlorophenolicum* also mineralizes 2,4,6-TCP (Cai and Xun 2002), some 2,4,6-TCP degraders, that do not carry *pcpC*, may also use PcpB and PcpA homologues for the degradation (Fig. 1.3).

### *1.3.3 2,4,5-Trichlorophenol Metabolic Pathway of B. Cepacia AC1100*

2,4,5-TCP is the first metabolic intermediate of 2,4,5-T degradation by *B. cepacia* AC1100 (Fig. 1.6) (Karns et al. 1983), and the 2,4,5-T degradation pathway has been deciphered by a combination of genetic and biochemical studies. Mutagenesis has produced two types of mutants: one completely loses the ability to degrade 2,4,5-T, and the other degrades it, but accumulates 5-chlorohydroxyquinol (Sangodkar et al. 1988; Haugland et al. 1991). Logically, the first type has a mutation in a gene responsible for the first step of the degradation pathway, and the second type has a mutation in the gene responsible for 5-chlorohydroxyquinol metabolism. Fortunately, several plasmid vectors can replicate in *B. cepacia*. The intact genes of *B. cepacia* AC1100 have been cloned, and transferred into the mutants. The cloned DNA fragments, that complement the mutants to grow on 2,4,5-T, have been mapped to identify the genes responsible for the first degradation step as well as for 5-chlorohydroxyquinol metabolism.

#### 1.3.3.1 2,4,5-Trichlorophenoxyactate Oxygenase (TftAB)

The 2,4,5-T oxygenase has an oxygenase component and a reductase component. It is the first enzyme of the 2,4,5-T degradation pathway of *B. cepacia* AC1100 (Fig. 1.6). The genes encoding the 2,4,5-T oxygenase component (*tftA* and *tftB*) have been cloned for their ability to complement the mutant that does not grow on 2,4,5-T (Hubner et al. 1998). The two genes *tftA* and *tftB* are homologous to the

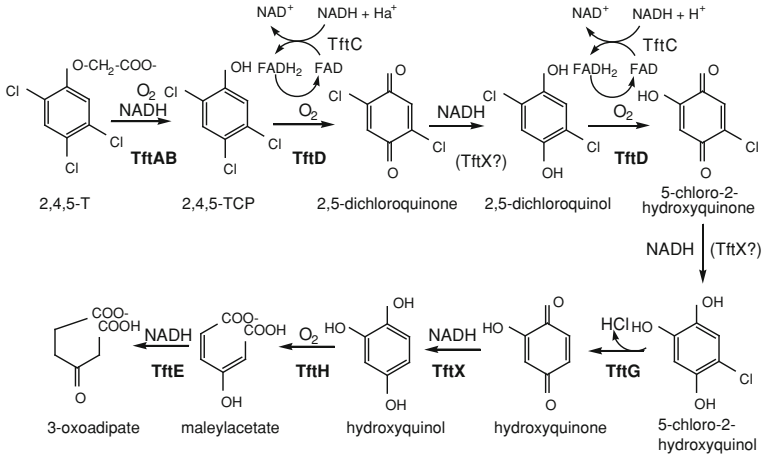


**Fig. 1.6** 2,4,5-Trichlorophenoxyacetate degradation pathway of *B. cepacia* AC1100. TftAB, 2,4,5-T terminal oxygenase; TftD, 2,4,5-TCP 4-monooxygenase; TftC, NADH:FAD oxidoreductase; TftX, quinone reductase; TftG, 5-chlorohydroxyquinol dehydrochlorinase; TftH, hydroxyquinol 1,2-dioxygenase; and TftE, maleylacetate reductase

$\alpha$- and $\beta$-subunits of the oxygenase component of ring hydroxylating dioxygenases (Hubner et al. 1998), but the gene encoding a reductase component is not next to *tftA* and *tftB*. Theoretically, the oxygenase component requires a reductase component to function. The 2,4,5-T oxygenase has been studied from *B. cepacia* AC1100, and it has an oxygenase component and a reductase component (Xun and Wagon 1995). The 2,4,5-T oxygenase component (TftAB) is a heterotetramer with an $\alpha_2\beta_2$ structure, and each $\alpha\beta$ unit has a Reiske-type [2Fe-2S] center, which gives the protein a reddish color with absorption peaks at 420 (shoulder) and 530 nm. The N-terminal sequences of the $\alpha\beta$ subunits match with those encoded by *tftA* and *tftB*. As expected, the oxygenase component alone has no activity, and it requires a reductase component to oxidize 2,4,5-T to 2,4,5-TCP and glyoxylate. The reaction is a typical monooxygenation reaction, different from the reaction typically catalyzed by most ring hydroxylating dioxygenases; however, it should not be a surprise, as some ring-hydroxylating dioxygenases can catalyze monooxygenation reactions towards certain substrates (Wackett et al. 1988). The reductase component has been only partially purified from *B. cepacia* AC1100 (Xun and Wagon 1995), and the corresponding gene has not been cloned.

### 1.3.3.2 2,4,5-Trichlorophenol 4-Monooxygenase (TftCD)

Mutations that block 2,4,5-TCP metabolism have not been obtained. The 2,4,5-TCP 4-monooxygenase is identified, purified, and characterized directly from *B. cepacia* AC1100 (Xun 1996). HPLC analysis showed that the cell extract consumes 2,4,5-TCP without any apparent end products. When 2,4,6-TCP is used