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**THE ANAEROBIC, PHOTOTROPHIC METABOLISM OF
3-CHLOROBENZOATE BY *RHODOPSEUDOMONAS PALUSTRIS***

by

Varsha Subhash Kamal

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

October 9th, 1992

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**THE ANAEROBIC, PHOTOTROPHIC METABOLISM OF
3-CHLOROBENZOATE BY RHODOPSEUDOMONAS PALUSTRIS**

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Carleton University

November, 1992

DEDICATION

**This thesis is dedicated to the memory of my mother,
who always wanted me to be "Doctor".**

To my father, who developed the quest of knowledge in me.

**And to my husband, without his encouragement and
understanding this was a dream.**

ABSTRACT

The degradation of chlorinated aromatic compounds by anaerobic bacteria is now known to be an important mechanism of bioremediation. This process has not previously been studied in the phototrophic bacteria. In this study a mixed phototrophic culture was found to metabolize 3-chloro-benzoate in the presence of benzoate under anaerobic, light conditions, following adaptation on a benzoate and 3-chlorobenzoate medium for 7 weeks. The dominant bacterial isolate, WS17, was identified as *Rhodopseudomonas palustris*. Radioisotopic studies showed ^{14}C -3-chlorobenzoate was converted by WS17 to $^{14}\text{CO}_2$ and cell biomass in the absence of oxygen and in the presence of a cosubstrate and light. The optimum conditions were pH 7.25, temperature 37°C and a cosubstrate concentration of 1mM benzoate. Cyclohexane carboxylate was able to replace the cosubstrate, benzoate. WS17 also metabolized 3-chlorobenzoate in the presence of pimelic acid, sodium acetate and sodium succinate, however the metabolic rate was reduced. Gas Chromatography Mass Spectrometry and High Pressure Liquid Chromatography indicated the intracellular presence of 3-chlorobenzoate and benzoyl-CoA. Cell free extracts produced benzoate and benzoyl-CoA. Stoichiometric release of chloride during the metabolism of 3-chlorobenzoate, and inhibition by the suspected intermediates cyclohexane carboxylate and pimelic acid, suggested a probable route of 3-chlorobenzoate metabolism via dehalogenation followed by steps similar to the

benzoate reductive ring fission pathway. Comparison of kinetic coefficients $\mu_{\max}$ and K_s for benzoate (1.658 mM/Day and 0.077mM respectively) and those for 3-chlorobenzoate (0.279 mM/Day and 0.1117 mM) showed higher affinity of WS17 for benzoate. Isolates from representative samples of various freshwater and wastewater ecosystems indicated widespread ecological distribution of *R. palustris* species and the common occurrence of the 3-chlorobenzoate metabolic phenotype. *R. palustris* WS17 was found to grow in mixed anaerobic cultures and retained its 3-chlorobenzoate degradation property. WS17 has a wide substrate range including 2-chlorobenzoate, 3-bromobenzoate, 4-hydroxy benzoate, vanillic acid, isovanillic acid, aniline and 3-chloroaniline. Due to its extended substrate range and widespread ecological distribution, the organism is expected to play a significant role in environmental pollution control.

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This thesis is incomplete without recognizing contributions by various people. First to mention should be Dr. Cam Wyndham, whose valuable guidance and constant support made this difficult task simpler and enjoyable at times. I am sincerely grateful to all those who helped me: To Dr. Sinclair and Dr. Pick for their critical reviews and useful suggestions which helped streamline the research; To The Association of Universities and Colleges of Canada for financial support; to my labmates Jim, Roberta, Cindy, Maysoon, Alisa and Michelle for the thoughts we exchanged and fun times we had; to Joan Mallett, Geza Kiss and Clem Kazekoff (University of Ottawa) for their technical assistance; and to everybody in the ELBA greenhouse for keeping the atmosphere really green.

Besides research, my stay in Ottawa was a wonderful time with my several friends who were always there when I needed them, nonetheless, Doday needs special mention. My special thanks are reserved for my uncle and auntie who are taking care of my baby in India, while I finish the degree. This research endeavour was a dream without encouragement and support from my husband. Finally, I should be finishing soon, as the sweet, smiling face and lovely big eyes of my little baby (Shikha/Rucha) are calling me home.

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CHAPTER 1
BACKGROUND, RATIONALE AND OBJECTIVES
FOR STUDIES UNDERTAKEN

CHLORINATED AROMATICS IN THE ENVIRONMENT

Chlorinated aromatic compounds enter the environment in substantial quantities from various industrial and agricultural sources (Chapman 1976). They are xenobiotic, recalcitrant compounds and tend to persist in the environment due to their stable chemical nature. The resonating bonds of the benzene ring of chloroaromatics are stabilized by highly electronegative chloride, yielding a molecule resistant to abiotic change. The size of the chlorine atom and the resulting steric hindrance of enzyme-catalyzed reactions may also contribute to the recalcitrance of these compounds. Although these are toxic, xenobiotic and recalcitrant compounds, a few microorganisms have evolved effective means of metabolizing halogen substituted aromatic rings. They employ radically different biochemical strategies (depending on the availability of oxygen) to destabilize the resonance of the benzene rings of these molecules. Under natural environmental conditions, both aerobic and anaerobic microbial processes are involved in the metabolism of chlorinated aromatics. In most aerobic processes, oxidation of the aromatic ring occurs at the expense of molecular oxygen, which serves as the terminal electron acceptor. Oxygen atoms are

usually incorporated directly into the aromatic ring in reactions that involve oxygenase enzymes, either monooxygenases or dioxygenases. Hydrolysis of chlorine substituents involving oxygen derived from water is also known to occur. In anaerobic processes, bacteria use inorganic electron acceptors to metabolize substrates in the absence of oxygen. Thus, NO_3 is reduced to N_2 or NH_3 ; SO_4^{2-} to S^{2-} ; and CO_2 to CH_4 in denitrifiers, sulfate reducers and methanogens, respectively.

AEROBIC AND ANAEROBIC DEGRADERS AND METABOLIC PATHWAYS

Although both aerobic and anaerobic microorganisms are responsible for the removal of halogenated recalcitrant compounds from the environment, only aerobic metabolism of aromatics has been intensively studied in many genera of bacteria (Bayly and Barbour 1984; Dagley 1986; Gibson and Subramanian 1984). The general mechanism of aerobic degradation involves hydroxylation of the aromatic ring at two adjacent carbon atoms, followed by *ortho*- (between the hydroxyl groups) or *meta*- (beside the hydroxyl groups) ring fission. These reactions result in the linearization of the aromatic molecule. The halogen substituents of the ring may be removed as a result of the initial ring hydroxylation, or may be removed during the subsequent metabolism of linearized metabolites, ultimately leading to the formation of intermediates of the Krebs citric acid cycle (Dagley 1986).

Biochemical mechanisms of anaerobic metabolism have been much less thoroughly examined, for reasons that include the difficulty of isolating pure cultures of anaerobes able to dehalogenate and grow on chlorinated aromatics, the difficulty of cultivating and maintaining these cultures once isolated, and the apparent lability of reductive aromatic catabolic enzymes. There are now many anaerobic dehalogenation studies in the literature dealing with consortia of methanogens and the sulfate and nitrate-reducing bacteria (Horowitz et al. 1983; Mohn and Tiedje 1992; Sharak Genthner et al. 1989a and b; Suflita et al. 1982; Tiedje et al. 1987; Urbanek et al. 1989). The intense activity in anaerobic culture studies has resulted in the isolation of a few dehalogenating anaerobes in pure culture. These include a few denitrifiers and a sulfate reducing bacterium: The denitrifying *Pseudomonas* species strain PN-1 reported by Taylor and Heeb (1972) and Taylor et al. (1979) and recently reclassified as *Alcaligenes xylosoxidans* sub-species *denitrificans* PN-1 by Blake and Hegeman (1967) as well as its mutant *Pseudomonas* PNS-1 have been shown to dehalogenate *ortho* and *para* fluorobenzoates and *ortho* chlorobenzoate; *Pseudomonas* species strain KB740 studied by Schennen et al. (1985) degrades 2-fluorobenzoate releasing stoichiometric amounts of halogen in the medium. Similarly, the sulfate-reducing bacterium *Desulfomonile tiedjei* DCB-1 isolated by Shelton and Tiedje (1984) dehalogenates 3-chlorobenzoate to yield chloride ions and benzoate. Benzoate

serves as a carbon and energy source for other bacteria when strain DCB-1 is grown in an anaerobic consortium. Thus, in the anaerobes studies to date, it is observed that the dehalogenation occurs prior to ring fission (Suflita et al. 1982). Reductive dehalogenation of the aromatic ring of 3-chlorobenzoate has recently been shown to yield energy in a form that is useful for the growth of strain DCB-1, despite the fact that this strain is unable to use the carbon and energy available in the product of the reaction, benzoate (Dolfing 1990).

While anaerobic dehalogenation has been studied in only a few isolates, the degradation of simple aromatic compounds has been studied extensively in several groups of anaerobes including nitrate and sulfate reducers, methanogens and photoheterotrophs (Berry et al. 1987; Bossert and Young 1986; Dutton and Evans 1967; Grbic-Galic and Vogel 1987; Harwood and Gibson 1988; Young 1984). The general mechanism of anaerobic metabolism of aromatics like benzoic acid involves reduction of the aromatic ring through hydrogenation, then addition of an hydroxyl group to the reduced ring, followed by ring cleavage in steps similar to the β -oxidation of fatty acids (Dutton and Evans 1969; Balba and Evans 1977).

PHOTOTROPHIC METABOLISM

The earliest studies of anaerobic biodegradation of aromatics were conducted with the photosynthetic bacterium

Rhodospseudomonas palustris. Dutton and Evans identified the key intermediates of anaerobic phototrophic metabolism of benzoate in *R. palustris* and suggested a reductive ring cleavage pathway through cyclohexane carboxylate and pimelic acid (Figure 1.1) (Dutton and Evans 1968b and 1969). The pathway can be considered in two parts. The first part is the reduction of the aromatic ring and the second part is a sequence of reactions similar to β -oxidation of fatty acids which involves alternate dehydrogenation and hydration. However, because in this case the substrate is a ring compound, instead of the usual release of a two carbon fragment (acetyl-CoA) at the end of the sequence, β -oxidation leads to breakage of the ring in the 1,2-position. Several researchers confirmed this mechanism using various approaches, including studies with metabolic mutants and cell-free systems (Guyer and Hegeman 1969; Dutton and Evans 1968a and 1970; Whittle et al. 1976). Enzyme studies contributed additional information that the intermediates in the pathway are conjugated to coenzyme A (Hutber and Ribbons 1983; Balba and Evans 1977; Harwood and Gibson 1986; Schennen et al. 1985; Williams and Evans 1975; Geissler et al. 1988).

For many years the study of anaerobic phototrophic degradation of aromatic compounds used benzoate as a model substrate. The range of substrates metabolized by *R. palustris* was recently extended to many phenolic, dihydroxylated and methoxylated aromatic acids as well as aromatic aldehydes;

FIGURE 1.1: PHOTOMETABOLISM OF BENZOATE IN *R. palustris*

making *R. palustris* one of the most versatile of the anaerobic bacteria with respect to aromatic degradation (Harwood and Gibson 1988).

Although *R. palustris* is now known to degrade a wide variety of substituted and unsubstituted aromatics, there has been no report on its ability to degrade haloaromatics. Harwood and Gibson (1988), reported the inability of chlorobenzoates to support the growth of *R. palustris* strains CGA001, CGA033 and CGA047. However, preliminary studies conducted by Wyndham (1987, unpublished observations) indicated that there was some potential for 3-chlorobenzoate dehalogenation during the growth of a mixed phototrophic culture in the presence of benzoate under anaerobic conditions in the light. This prompted an interest in studies of the anaerobic phototrophic metabolism of chlorinated aromatic compounds and was the instigation for the research reported here.

RESEARCH OBJECTIVES

Part of the inspiration for the research presented in this thesis is the observation that photosynthetic bacteria play a significant role in the dissimilation of organic matter in natural aquatic environments and in many industrial wastewater treatment systems. The photosynthetic bacteria belonging to the Family Proteobacteria have a widespread ecological distribution and an ability to metabolize various benzenoid compounds (Sleat and Robinson 1984). Although

degradation of aromatic compounds is considered important for the control of aromatic pollutants, degradation of chloroaromatics is of particular interest because of their ubiquitous and problematic nature.

The following research work is an attempt to deal mainly with one chlorinated aromatic compound, 3-chlorobenzoate, and related aromatics. The following objectives have been achieved:

1. Enrichment of a mixed, phototrophic, dehalogenating culture and its characterization.
2. Enumeration of requirements of the isolate *Rhodopseudomonas palustris* WS17 for 3-chlorobenzoate mineralization.
3. Determination of the mechanism of 3-chlorobenzoate metabolism in *R. palustris* WS17.
4. Kinetic studies of 3-chlorobenzoate degradation in *R. palustris* WS17.
5. Investigation of the survival of *R. palustris* WS17 in mixed bacterial cultures and its ability to metabolize other substituted aromatics.
6. Investigation of the ecological distribution of *Rhodopseudomonas* species having the 3-chlorobenzoate metabolic phenotype.

General methods used throughout the thesis research are described in Chapter 2. Chapter 3 describes the growth of a

mixed phototrophic culture and the isolation, identification and characterization of the dehalogenating organism *R. palustris* WS17 obtained after enrichment from this mixed culture. Chapter 4 presents evidence for the uptake and mineralization of 3-chlorobenzoate by WS17 and describes the requirements of this organism for 3-chlorobenzoate metabolism. Various approaches adapted for understanding the mechanism of 3-chlorobenzoate metabolism are described in Chapter 5. This Chapter also includes an investigation of the kinetics of 3-chlorobenzoate degradation in WS17. Experiments conducted to assess the survival of WS17 in mixed bacterial cultures and its ability to metabolize a wide variety of substituted aromatics are described in Chapter 6. This Chapter also includes studies of the ecological distribution of *Rhodopseudomonas* species expressing the 3-chlorobenzoate metabolic phenotype. The final chapter summarizes the main conclusions of this research.

CHAPTER 2

BASIC METHODS AND MATERIALS

This chapter outlines the materials and methods of a general nature used in this research. Detailed materials and methods that are particular for the experimental work described in Chapters 3 to 6 are contained in those Chapters.

BACTERIAL STRAINS

The following bacterial cultures were used during these studies:

1. A complex mixed anaerobic culture of bacteria dominated by phototrophic purple sulfur and non-sulfur bacteria was kindly provided by Dr. Wilfred Hahn (Activite Biotechnology Development Corporation, Ottawa, Ontario, Canada). This culture was enriched from sediments with no prior exposure to halogenated aromatics and contained approximately 10^8 phototrophic bacteria and 10^7 aerobic and facultative anaerobic heterotrophic bacteria per ml. The exact composition of the mixed culture can not be described due to proprietary rights of the organization.

2. The pure culture isolates obtained from the aforementioned mixed culture through enrichment on benzoate and 3-chlorobenzoate: *Rhodopseudomonas palustris* WS17, *Rhodospirillum* species WS9 and an unidentified purple phototroph WS8.

3. Freeze dried sample of type strain of *R. palustris* (ATCC 17001) obtained from the American Type Culture Collection and stored at -70°C .

4. The pure culture isolates of *Rhodopseudomonas* species obtained from various environmental samples using ecocore tube technique and dilution plating on a selective medium containing 3-chlorobenzoate: *R. palustris* Rps-5 (isolate from pond water of a greenhouse in Carleton University, Ottawa); JL78, JL86, JL88, JL94, JL96 and JL98 (isolates from Jacks lake, Peterborough, Ontario); RS51, RS52, RS71 and RS72 (isolates from sediments of Rideau river on Carleton University campus, Ottawa).

5. *R. palustris* strains CGA001, CGA033 and CGA047 were provided by Dr. Jane Gibson (Cornell University, Ithaca, New York, USA). CGA001 is a wild type isolate originally isolated by Dr. S. Taniguchi, whereas CGA033 and CGA047 are mutants of CGA001. CGA033 is unable to grow aerobically with 4-hydroxybenzoate but is wild type with respect to anaerobic growth. Strain CGA047 is an anaerobic mutant and cannot grow on either benzoate or 4-hydroxybenzoate under anaerobic conditions but has unimpaired aerobic growth capabilities.

GROWTH MEDIA AND CULTURE CONDITIONS :

The complex medium P was used for cultivation of the mixed culture; isolates of phototrophs : WS8, WS9 and WS17 obtained after enrichment of the mixed culture; *R. palustris*

CGA001, CGA033, CGA047 and the type strain ATCC 17001. Medium P contained (grams per litre): K_2HPO_4 , 1.71; NaH_2PO_4 , 0.34; NH_4Cl , 0.5; $MgSO_4$, 0.4; $CaCl_2$, 0.05; $NaCl$, 0.5; Yeast Extract (Difco), 0.5; and 10 ml of a 100x concentrated trace elements stock solution containing (grams per litre): $Na_2EDTA \cdot 2H_2O$, 12.0; $NaOH$, 2.0; $ZnSO_4 \cdot 7H_2O$, 0.4; $MnSO_4 \cdot 4H_2O$, 0.4; $CuSO_4 \cdot 5H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 3.0; Na_2SO_4 , 5.0 and $NaMoO_4 \cdot 2H_2O$, 0.1. Benzoate and 3-chlorobenzoate were added at various concentrations as carbon and electron sources and the pH was adjusted to 7.2. Screw cap bottles or tubes were filled, autoclaved, and finally topped up to avoid oxygen in the headspace.

During initial experiments on the acclimatization of a mixed phototrophic culture, medium P was supplemented with 0.5 grams per litre of sodium succinate as an additional source of carbon and electrons.

The liquid cultures were grown in medium P at 26°C in batch or in continuous culture systems (described in the following section) under anaerobic conditions in the presence of light. The cultures were illuminated with a 250 W infrared lamp, at an approximate distance of 1 meter, with an orange filter (infrared to red wavelength transparent) placed between the lamp and the cultures. Intensity of the light reaching culture bottles was measured with Li-Cor quantum light detector Q10829. Readings taken at various distances in the incubation area showed that the intensity was in the range of

14 to 20 $\mu\text{mole per second per square meter}$ ($\mu\text{mole/s/m}^2$), which is nearly 41 to 59% of the day light intensity ($34 \mu\text{mole/s/m}^2$). The onset of anaerobic conditions in the batch and continuous cultures was assessed by observing the induction of culture pigments (carotenoids and bacteriorhodopsin) indicative of phototrophic growth. Many of the non-sulfur, phototrophic bacteria are capable of heterotrophic growth under aerobic conditions, and must use up available oxygen before starting to grow by phototrophic means. Phototrophic growth is known to occur only under anaerobic conditions, in the presence of light (Brock and Madigan 1988). This activity was relied upon to eliminate the traces of oxygen remaining in the media after autoclaving.

Occasionally, Yeast Extract medium (ATCC medium 112) was used to obtain 18 or 24 hr liquid cultures with high density. The medium contained the following (in grams per litre); K_2HPO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 and Yeast Extract (Difco), 10.0.

To obtain pure culture isolates and for colony counts, cultures were grown on agar (1.8%)-solidified medium P or Yeast Extract medium (ATCC medium 112). The agar plates were incubated in Gas-Pak cylinders (BBL Microbiology Systems, Cockeysville, MD) containing a catalytically generated atmosphere of hydrogen and carbon dioxide at 26°C . The cultures were illuminated with a 250 W infrared lamp as described for the liquid cultures.

The following physical systems were employed for the

growth of batch and continuous cultures:

For anaerobic batch cultures (Figure 2.1), 750 ml or 1500 ml capacity screw capped culture bottles (Gibco or Wheaton) were used. After inoculation the bottle was filled to the top with sterilized culture medium and was sealed with a rubber stopper to prevent any diffusion of oxygen from the external environment. To facilitate sampling from this sealed bottle two syringes were inserted through the stopper. One of the syringes was empty and was used to withdraw sample from the culture bottle. The other syringe was filled with the medium which acted as a reservoir for replacing the medium equal to the sample volume withdrawn.

For anaerobic continuous cultures (Figure 2.2), a 1500ml capacity continuous culture flask was used. The lid of this flask was equipped with various ports for inoculation, medium feed, pH adjustment, temperature measurement, gaseous injection, mixing and sampling. Anaerobic conditions were maintained by flushing the head space with a $\text{CO}_2:\text{N}_2$ (9:1) mixture prior to the start of experiment and through continuous flow of $\text{CO}_2\text{-N}_2$ mixture in the culture vessel at a very low rate during the entire experimental period. Sampling was conducted through the port provided in the lid using an aspiration sampling assembly after complete mixing of the contents.

FIGURE 2.1 : ANAEROBIC BATCH CULTURE SYSTEM

FIGURE 2.2 : SCHEMATIC OF ANAEROBIC CONTINUOUS CULTURE

GROWTH MEASUREMENT

Optical Density Measurement At 660 nm.

Initially, growth in liquid cultures was monitored by measuring the optical density at a wavelength of 460 nm. However, erroneous results were obtained by this method as the pigment production and growth were observed to follow a similar trend only during the logarithmic growth phase. Growth was subsequently measured at a wavelength of 660 nm. This wavelength, rather than the usual 600 nm, was selected because the absorption spectrum for pigments produced by the photosynthetic bacteria has a minimum at 660 nm. Therefore, absorption by cell suspensions at this wavelength should primarily be a result of light scattering by the cells. For all the experiments included in this thesis culture growth was determined by measurement of optical density at 660 nm by using a Beckman model 26 UV/visible spectro-photometer.

Total Protein Concentration.

Growth was also determined by measuring total protein concentrations using the principle of protein-dye binding (Bradford 1976). For protein determinations, 1 ml of culture fluid was transferred to a microfuge tube and cells were harvested by centrifuging at 13000 rpm for 10 minutes. The harvested cells were washed with saline and suspended in 0.1 ml of sterile distilled water. The cell suspension was extracted with 5% (w/v) trichloroacetic acid and cell residue was heated for 10 minutes at 95°C in 0.1N NaOH (Harwood and

Gibson 1986). The mixture was cooled to room temperature and a dye binding assay procedure was employed to estimate protein concentration (Bradford 1976).

CHEMICAL CONCENTRATION DETERMINATION BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Samples from cultures were collected at intervals, centrifuged twice to remove cells, and the supernatant was analyzed immediately or stored at -20°C until analyzed. High pressure liquid chromatography (HPLC) analysis was used to estimate substrate disappearance during anaerobic growth. A Waters isocratic system 501 with a Nova Pak C-18 reverse phase column (7 cm, 4 μm packing) and a UV detector (Lambda-Max Model 481 LC) set at 254 nm wavelength was used for analysis. Samples of 100 μl of the culture supernatant were injected into a 20 μl injection loop. The mobile phase was a 1:1 mixture of methanol and 1% acetic acid for all analyses unless otherwise mentioned. The flow rate was adjusted to 0.8 ml per minute and the run time was 15 minutes. Concentrations of substrates were determined by comparison of chromatograph peak areas with injections of standards. Multiple injections of the same standards indicated that variation between samples during one machine operation period (usually one day) was $\pm 5\%$. There was greater variation between samples analyzed during separate machine operation periods (day to day variation) due to changes in column pressure and detector sensitivity. This

variation was $\pm 10\%$.

The above methods were standard for the entire study. Specific materials and methods are given in detail in each of the Chapters dealing with the results of particular experiments (Chapters 3 to 6).

CHAPTER 3
ENRICHMENT OF A MIXED, PHOTOTROPHIC, DEHALOGENATING CULTURE
AND ITS CHARACTERIZATION

INTRODUCTION

Phototrophic bacteria have diverse metabolic abilities. The purple non-sulfur group of phototrophs, specifically the several species belonging to the family *Rhodospirillaceae*, are characterized by their ability to grow on a variety of aliphatic as well as simple aromatic compounds. Bacteria of this family are able to grow using these substrates as a sole carbon source, both anaerobically in the light by photosynthetic means and aerobically in the dark by respiration (Dutton and Evans 1978). Thus in anaerobic environments with low light intensities these organisms may be contributing significantly towards the removal of aromatic pollutants. With this rationale in mind a mixed bacterial culture, enriched from sediments and dominated by phototrophic bacteria, was expected to contain anaerobic degraders of aromatic compounds and hence was used for studying anaerobic degradation of the halogenated aromatic compound, 3-chlorobenzoate.

MATERIALS AND METHODS

ACCLIMATIZATION

Acclimatization of a mixed anaerobic culture was the first step to obtain dehalogenating populations. This was achieved through cultivation of the mixed anaerobic population in batch culture. Medium P containing 1.0 mM benzoate, 0.5 mM 3-chlorobenzoate and 0.05% sodium succinate as carbon and electron sources was inoculated and incubated under anaerobic conditions in presence of light (as described in Chapter 2). Disappearance of the substrates was monitored using HPLC analyses on culture supernatants and the changes in microbial population were noted by intermittent microscopic observations. When the substrate benzoate was exhausted, 10 percent of the culture was transferred to fresh medium P. Over a period of 5 weeks two transfers were done.

ISOLATION OF DEHALOGENATING PHOTOTROPHS

The acclimatized culture was then used to obtain pure culture isolates of bacteria capable of 3-chlorobenzoate degradation. The mixed culture was plated on solidified (1.8% agar) medium P with no carbon source dissolved in the medium. 3-Chlorobenzoate (0.004 gm) was applied to the surface of solidified medium P at a point near the edge of a plate. These plates were incubated anaerobically in the light. Growth from these auxanography plates was resuspended in sterile distilled water and replated on medium P with dissolved 0.5 mM 3-

chlorobenzoate. Two types of red pigmented colonies (1.5-2.0 mm and pin point) were subcultured using dilution plating technique. The process was repeated several times to get pure culture isolates, later named WS8, WS9 and WS17. These isolates were further subjected to 3-chlorobenzoate degradation studies in liquid cultures to determine the strains responsible for the dehalogenation activity of the mixed culture.

ISOLATE CHARACTERIZATION

The phototrophic isolate WS17 was further identified and characterized based on morphological, metabolic and genetic criteria.

General Characterisation.

The culture was characterized by determining cell shape and size, Gram staining properties, motility, presence or absence of flagella and flagellar type. The liquid broth cultures of WS17 at different ages were subjected to microscopic observations to reveal the shape and motility of WS17. An ocular scale was used to determine the cell size. WS17 cells fixed on a clean glass slide were stained with crystal violet and safranin using Grams staining technique and observed under the light microscope. The presence of flagella was determined with Gray's mordant and Leifson's staining technique.

Gray's mordant was made by mixing 5 parts of saturated

potassium aluminum sulphate, 2 parts 20% tannic acid, 2 parts saturated mercuric chloride and 0.8 parts saturated alcoholic basic fuchsin. The mordanting solution was applied to the edge of a wet mount and the cells observed by phase contrast illumination on a Zeiss photomicroscope-II with an oil immersion objective. Liefson's staining technique was later used to confirm the initial observations. Liefson's stain was prepared by mixing 1.2% alcoholic basic fuchsin, 3% tannic acid and 1.5% sodium chloride in equal parts. The mixture was allowed to settle for 24 hours before use. A 1ml sample of the culture ($OD_{660}=0.438$) was centrifuged at 5000 rpm for 5 minutes. The pellet was washed with tris-EDTA (pH 7.2) and resuspended in a small volume of 1% formalin. This suspension was applied to a clean glass slide and allowed to dry. This dry mount was covered with Liefson's stain and allowed to stand for 15 minutes (when the surface of the stain appeared covered with precipitate). The slide was rinsed in distilled water, dried and examined by phase contrast illumination under oil immersion.

Electron Microscopy.

Further details of cell structure were studied using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Transmission electron microscopic observation of ultrathin sections of WS17 was done following fixation, embedding and sectioning of mid-exponential growth phase

cells. The cells were first fixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) for 2 hours, washed twice in phosphate buffer, postfixed with 1% osmium tetroxide for another 2 hours and washed again with the same buffer. This was followed by dehydration through a series of 25%, 50%, 70%, 90% ethanol in water washes and final washes with absolute alcohol and propylene oxide. The cells were stained with 1% uranyl acetate during the 50% ethanol in water wash and finally embedded in epon after the dehydration process was complete. The sections obtained with a Reichert OM U2 (Austria) microtome were observed with a Phillips 400 transmission electron microscope.

For scanning electron microscopy 1 ml of a 10^{-2} diluted broth culture of WS17 was filtered through a 0.2 μm filter (Millipore Corporation). The filter was placed on a wet piece of blotting paper to prevent it from drying. A small strip containing evenly distributed WS17 cells was cut from this filter and was placed on a sample holder. The sample was further subjected to freeze drying treatment under liquid nitrogen prior to its transfer to the sample chamber and was finally observed on the screen at 1 kilovolt using Jeol JSM 6400 scanning electron microscope.

Pigment Analyses.

Pigment analyses of an exponentially growing culture of *R. palustris* WS17, the type strain ATCC 17001 and the mixed phototrophic culture were conducted as follows. Cells were

harvested by centrifugation at 10,000 rpm for 15 minutes. The pellets were washed with 10 mM TEA-PO₄ buffer (triethanolamine phosphate) of pH 7.2 and the pigments were extracted by vortexing the pellet with 7 parts methanol and 2 parts acetone mixture. The extraction process was repeated three times (till the pellet was colourless) and the extracts for all the three batches were mixed together. Finally, the pigment analyses were conducted on these extracts using a Hewlett Packard 8452A Diode Array spectrophotometer by scanning between 820 nm and 190 nm (Cohen-Bazire et al. 1966).

Antibiotic Resistance.

Antibiotic resistance properties of WS17 were tested with ampicillin, chloramphenicol, kanamycin, streptomycin and tetracyclin in various concentration ranges. The culture tubes with 20 ml of medium P containing antibiotic were inoculated with 200 µl of exponentially growing WS17 culture (OD₆₆₀= 1.311). The tubes were then incubated at 26°C under anaerobic conditions and in the presence

of light for 24 hours. Presence or absence of turbidity was noted at the end of the incubation period.

Metabolic Activity.

The ability of WS17 and ATCC 17001 to metabolize a range of aliphatic and aromatic compounds was tested using the following two techniques.

1. Biolog Microplates (BIOLOG Inc. Hayward, California)

are designed with 96 wells to test the ability of a microorganism to utilize (oxidize) 95 different carbon sources of various groups. One of the wells serves as a control with no substrate added to it (Appendix I presents the names and positions of various substrates on the microplates). The respiration by live cells is indicated colorimetrically by the reduction of a tetrazolium dye to produce a purple precipitate (an uncharacterized polymeric compound). The organic compound in each well will only yield a purple precipitate if it serves as a source of electrons for respiratory growth. The test yields a metabolic fingerprint of the inoculated organism under aerobic conditions. To prepare the inoculum for Biolog microplates, the isolate WS17 and ATCC 17001 were streaked on yeast extract medium agar (ATCC medium 112) and grown for 18 hours at 26°C under anaerobic conditions in Gas-Paks. Growth was removed from agar plates by rolling a sterile swab over colonies. The cells were suspended in 1 ml of sterile saline (0.85%) by twirling the swab against the inside surface of the microfuge tube. The suspension was centrifuged at 5,000 rpm for 2 minutes in an Eppendorf centrifuge. The pellet was resuspended in 20 ml of sterile saline to obtain the required inoculum turbidity. The suspension was vortexed and transferred to an inoculum tray. 100 μ l of this inoculum was pipetted into each well of the Biolog microplates using a multipipetter and incubated for 24 hours under aerobic conditions at 26°C in the presence of light and at 30°C in the

dark. The control well showed no coloration. The results were noted by comparing a colour reaction in any other well with that of the control.

2. Auxanography was used to assess both the capacity of an organic substrate to support growth and the relative toxicity of the substrate. The isolate WS17 was grown anaerobically with 1 mM sodium succinate as carbon and electron source. The cells were harvested by centrifuging at 7,000 rpm for 15 minutes and washed twice with a sterile 0.85% NaCl solution. The saline suspension of these washed cells (absorbance 0.403 at 660 nm) was used as an inoculum for auxanography by mixing 100 μ l with 20 ml of defined medium P (medium P without yeast extract) supplemented with p-aminobenzoic acid (0.15 mM); biotin (0.15 mM) and 1.5% (w/v) agar, and incubated at 45°C. The medium was then immediately poured into a petri dish and allowed to solidify. The substrates to be tested (Table 3.3) were applied as solids to the surface of the solidified medium at a point near the edge in separate plates. A control plate did not have any carbon source. The results were noted as presence or absence of growth as an arc with an inhibition zone close to the point of substrate addition, or a uniform lawn all over the plate.

Plasmid Screening.

Plasmid screens were performed on various strains of *R. palustris* using a quick, large scale plasmid preparation method of Krieg and Melton (Harvard University) (Promega

technical bulletin, Promega Biotec, # 009). The cells were harvested from 250 ml of culture by centrifugation at 6,000 rpm for 10 minutes. The pellet was washed with tris-EDTA (10 mM, pH 7.2) and was resuspended in 6 ml of freshly prepared solution I containing 25 mM tris-HCl, 10 mM EDTA, 15% sucrose and 5 mg per ml lysozyme. The suspension was incubated for 20 min. on ice. To this, 12 ml of solution II containing 0.2 M NaOH and 1% SDS was added and mixed thoroughly by inversion. The mixture was incubated for 10 min. on ice. At the end of incubation period 7.5 ml of 3 M sodium acetate (pH 4.6) was mixed with this lysate and the mixture was further incubated on ice for 20 min. Cell debris were removed by centrifugation at 10,000 rpm for 15 minutes. To the supernatant 50 μ l of RNase (1 mg per ml) was added and incubated for 20 min. at 37°C to digest RNA. DNA was extracted with phenol:chloroform (1:1) mixture and precipitated with 2 volumes of ethanol by cooling overnight at -20°C. The pellet of precipitated nucleic acids was separated by centrifugation at 10,000 rpm for 20 minutes. DNA was further purified from RNA and reprecipitated as follows. The pellet was dissolved in 1.6 ml of H₂O. To this solution 0.4 ml of 4 M NaCl and 2 ml of 13% PEG (polyethylene glycol) were added and the mixture was incubated on ice for 1 hour. A pellet of the clean DNA was separated by centrifugation at 10,000 rpm for 10 minutes and washed with 70% ethanol. The pellet was finally dissolved in 100-200 μ l of H₂O or 10 mM Tris 1 mM EDTA buffer. The DNA was loaded with a

blue electrophoresis running dye (0.025% bromophenol blue and 0.025% xylene cyanol FF) on a 0.7% agarose gel. Electrophoresis was conducted overnight at 10 volts.

The procedure was scaled down to a 5 ml volume of starting culture for plasmid isolations from some strains of *R. palustris*. For WS17, as the routine protocol was unable to isolate plasmid DNA, several variations were tried. For example, 100 ml of culture was treated as if starting from a 250 ml culture. Also the lysozyme concentration or the duration of incubations were varied. Other procedures used to screen for plasmid content included the in-well lysis protocol of Eckhardt (Eckhardt 1978) and a revised Crosa Falkow method (Wyndham and Straus 1988a).

Restriction Digestion

Plasmid preparations of *R. palustris* ATCC 17001 were subjected to restriction digestion with enzymes *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III. The digestion mixture was as follows. 4 μ l DNA (2.2 μ g per μ l) + 14 μ l milli-Q-water + 2 μ l buffer + 1 μ l enzyme (12 units per μ l for *Hind*III and 10 units per μ l for the rest). The mixture was incubated for 2 hours at 37°C in a waterbath. At the end of the incubation period 6 μ l stop solution (7 M urea, 50% sucrose, 50 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol FF, pH 7.0) was added and 25 μ l of this digest was loaded on a 0.7% agarose gel. Electrophoresis was conducted overnight at 10 volts.

RESULTS AND DISCUSSION

ACCLIMATIZATION OF MIXED PHOTOTROPHIC CULTURE

The degradation of benzoate and 3-chlorobenzoate by the mixed culture of photosynthetic bacteria under anaerobic conditions and in the presence of light is presented in Figure 3.1. Under these conditions benzoate degradation by the mixed culture began immediately, with 0.523 mM of benzoate being degraded completely within 9 days in the initial culture (culture A). Following the first transfer i.e. in culture B, 0.972 mM benzoate was degraded within 7 days. The maximum degradation rates were calculated from the data for substrate disappearance as the maximum slope of tangent to the degradation curve. The results show that the maximum benzoate degradation rate increased with transfer from approximately 0.12 mM per day in the initial culture (culture A) to 0.34 mM per day in the third culture (culture C). 3-Chlorobenzoate metabolism did not occur in these initial transfers. However, degradation of 3-chlorobenzoate occurred after the culture was acclimatized for nearly 6 weeks (in culture C). The maximum 3-chlorobenzoate degradation rate in this culture was found to be 0.07 mM per day. This acclimatized culture on further transfer to a fresh medium P (culture D) showed an increase in the maximum rate of 3-chlorobenzoate degradation to 0.13 mM per day. The results for culture C show that 3-chlorobenzoate degradation occurs only after benzoate is completely

**FIGURE 3.1: BENZOATE AND 3-CHLOROBENZOATE DEGRADATION
BY A MIXED CULTURE DURING ACCLIMATIZATION PROCESS**

Closed circles represent 3-chlorobenzoate concentrations and open circles, benzoate concentrations (n=1). Vertical lines correspond with the day cultures were transferred to fresh medium.

metabolized. In the next transfer conducted with the acclimatized mixed culture (culture D) simultaneous degradation of benzoate and 3-chlorobenzoate occurred, most probably because the culture was enriched for 3-chlorobenzoate degrading bacteria and these cells possessed an active enzyme system for the degradation of 3-chlorobenzoate.

The composition of the mixed culture changed during adaptation to chlorobenzoate. Photomicrographs (Figure 3.2, A) taken at the beginning of the acclimatization period clearly indicated a well mixed population of diverse bacteria with variable morphologies including cocci, bacilli, dumbbell shaped rods and spirilli etc. However, as the acclimatization period was progressing this culture showed an increase in the number of highly motile rod shaped bacteria and rosette clusters. This was a qualitative estimation based on intermittently conducted microscopic observations of the mixed culture at various stages of acclimatization. An attempt to support this observation through quantitative estimation using the plate count technique was not successful. As the cells formed clusters or rosettes, the plate count was not a true representation of the number of cells in the culture. A photomicrograph taken after two transfers of the culture (culture C) (Figure 3.2, B) shows enrichment of the purple non-sulfur group of bacteria, in particular cell types that resembled *R. palustris* in morphology and in the production of swarmer cells. This observation supports the fact that members

**FIGURE 3.2 : ENRICHMENT OF THE MIXED BACTERIAL CULTURE
UNDER ANAEROBIC, PHOTOTROPHIC CONDITIONS**

a, Photomicrograph taken at the beginning.

b, Photomicrograph after culture enrichment.

of the family *Rhodospirillaceae* are involved in the degradation of aromatic compounds.

CHARACTERIZATION OF THE DEHALOGENATING CULTURE

The acclimatized culture when plated on ATCC medium 112 and incubated anaerobically, yielded 14 different types of colonies depending on the colony shape, size and pigmentation. From these the red pigmented colonies of two types (pin point and 1-2 mm) were of interest and were selected for further work. The red pigmented colonies of both types were found to be mixed cultures of three different morphological forms; cocci, spirilli and highly motile club shaped rods clumping together and forming rosettes. Pure cultures of these three types of cells were obtained through repeated dilution plating and were designated as WS8, WS9 and WS17 (Figures 3.3, A, B and C respectively). These were subcultured several times to ensure purity and were further characterized. WS8 was a coccus which grew anaerobically in the presence of light on yeast extract medium 112. WS9 was a *Rhodospirillum* species as determined by anaerobic, phototrophic growth on ATCC medium 112, cell morphology and motility. WS17 was able to grow on both medium P supplemented with 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate and ATCC medium 112. WS17 was further characterized using several criteria and identified as a strain of *R. palustris*.

The isolates obtained from the mixed phototrophic culture were tested for 3-chlorobenzoate degradation under anaerobic

FIGURE 3.3 : ISOLATES OF THE MIXED CULTURE

- a.** WS8, an unidentified coccus
- b.** WS9, a *Rhodospirillum* species
- c.** WS17, a *Rhodopseudomonas palustris* strain
- d.** SEM of WS17 showing a cluster

conditions using liquid medium P with 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate in one set and only 0.5 mM 3-chlorobenzoate in the other set. Optical density at 660 nm for all the isolates at the beginning of the experiment were approximately equal (OD_{660} range was 0.091 to 0.96). Batch culture systems were employed for this purpose and the cultures were grown and monitored for substrate disappearance as described in Chapter 2. The substrate utilization pattern for these organisms is presented in Figure 3.4A and B. Isolate WS9 (*Rhodospirillum* species) was unable to degrade benzoate as well as 3-chlorobenzoate. *R. palustris* WS17 metabolized most of the benzoate in 4 days and nearly 0.35 mM 3-chlorobenzoate in 8 days. The phototrophic coccus, WS8, apparently metabolized 0.13 mM benzoate in 5 days and from 0.1 to 0.15 mM of 3-chlorobenzoate in 9 days. This slow degradation of 3-chlorobenzoate by WS8 was observed in the presence (Figure 3.4A), and in the absence of benzoate (Figure 3.4B). These results, combined with the qualitative observations of the dominant cell morphologies in the acclimatized mixed culture, suggested that *R. palustris* WS17 was an important dehalogenating organism in the community.

The newly explored property of 3-chlorobenzoate degradation for *R. palustris* WS17 has extended the range of aromatic substrates for photosynthetic bacteria. Many substituted and unsubstituted aromatics have been reported to act as substrates for *R. palustris*, but these did not include

FIGURE 3.4 A and B**A: METABOLISM OF 3-CHLOROBENZOATE AND BENZOATE
BY ISOLATES OF THE MIXED CULTURE**

A legend to the symbols used for the different isolates and different substrates metabolized is given in the figure (n=1).

**B: METABOLISM OF 3-CHLOROBENZOATE BY ISOLATES
IN ABSENCE OF BENZOATE.**

A legend to the symbols used for the different isolates is given in the figure (n=1).

halogenated compounds (Harwood and Gibson 1988).

Acclimatization as described in this chapter may be required for efficient expression of the chlorobenzoate degradative phenotype, and this may account for the failure of others to detect chloroaromatic metabolism in *R. palustris*. The results of this and subsequent chapters have been communicated in a recent publication (Kamal and Wyndham 1990).

CHARACTERIZATION AND IDENTIFICATION OF WS17

The chlorobenzoate degrading strain of *R. palustris* obtained from the acclimatized mixed culture was characterized and identified using morphological, metabolic and genetic criteria. The results for various experiments were compared with those of the American Type Culture Collection (ATCC) strain of *R. palustris* (ATCC 17001) to confirm the identity of WS17.

General Characterization

Microscopic observation of WS17 cultures showed Gram-negative, rod shaped bacteria, 0.7-0.9 μm wide and up to 2.0 μm long. Cells were motile by means of subpolar flagella. The cells in older cultures formed rosette clusters in which the individual cells were adhering to each other at their flagellated poles (Figure 3.3 C). This arrangement of cells for the WS17 culture is a typical characteristic of the *R. palustris* type strain ATCC 17001. WS17 reproduced by the process of budding. This differentiating life cycle consists of a mother cell elongating to form a tube, 1.4-1.9 μm long,

at the pole opposite to that bearing a flagellum. The end of the tube grows into a daughter cell, thus producing a bumbbell shaped organism. The log phase cultures typically exhibit a high proportion of these budding cells. Finally the daughter cell is released in the medium with asymmetric division. This daughter cell, called a swarmer cell, is coccoid or slightly elongated in shape and is highly motile.

Transmission electron micrographs of WS17 in longitudinal section showed the lamellar structure of internal photosynthetic membranes lying parallel to and underlying the cytoplasmic membrane (Figure 3.5, A). This is a distinguishing character of *Rhodopseudomonas* species. The dark spots most probably indicated the presence of carboxysomes (organelles responsible for the production of carboxylase enzyme, required for fixation of CO₂). The Gram negative nature of the WS17 cell wall was further confirmed by a transmission electron micrograph of its transverse section (Figure 3.5, B). This shows a typical double membrane cell wall structure consisting of the cytoplasmic membrane and an outer membrane sandwiching a peptidoglycan layer.

The scanning electron microscopy of WS17 was an attempt to find out the cause of attachment of cells in a rosette cluster. Although details of these clusters were not revealed, SEM (Figure 3.3, D) shows a typical cell arrangement for *R. palustris*.

FIGURE 3.5 : TRANSMISSION ELECTRON MICROGRAPH OF

***R. palustris* WS17**

a. Longitudinal section of WS17

CS, probably carboxysomes

PM, photosynthetic membrane

b. Transverse section of WS17

CM, cytoplasmic membrane

OM, outer membrane

PG, peptidoglycan

FIGURE 3.6A : SPECTROPHOTOMETRIC ANALYSIS OF PIGMENT EXTRACTS OF *R. palustris* WS17

FIGURE 3.6B : SPECTROPHOTOMETRIC ANALYSIS OF PIGMENT EXTRACT OF *R. palustris* 17001

FIGURE 3.6C : SPECTROPHOTOMETRIC ANALYSIS OF PIGMENT EXTRACT OF
MIXED PHOTOTROPHIC CULTURE

Scanning spectroscopy of acetone-methanol extracts of WS17, ATCC 17001 and the acclimatized mixed culture indicated in each case, two main absorption zones: from 320 to 540 nm and from 740 to 800 nm. Spectrophotographs (Figures 3.6 A, B, and C) for pigment extracts of these cultures show striking similarity. Absorption maxima for an axenic culture of WS17 at 330, 370, 400, 425, 450, 475, 505, 590, 695 and 770 nm match with those of *R. palustris* ATCC 17001, thus supporting the identification of WS17 as *R. palustris*. Also the match between WS17 and mixed culture spectra confirms the microscopic evidence that WS17 was the dominant phototroph in the acclimatized culture (Culture C).

Knowledge of the antibiotic resistance of a microorganism is useful for its selective enrichment, for its maintenance as an axenic culture and also for genetic studies. For determination of the antibiotic resistance properties of WS17, five antibiotics; ampicillin, chloramphenicol, kanamycin, streptomycin and tetracyclin were tested over various concentration ranges from 0 to 200 μg per ml of medium P (Table 3.1). WS17 was found to be sensitive to kanamycin for the whole range tested and to streptomycin above 100 μg per ml concentration. However, it was resistant to other antibiotics. Kanamycin sensitivity of WS17 is an important property for its genetic manipulation, since transposons like Tn5 bearing kanamycin resistance genes have proven invaluable in mutagenesis studies with related phototrophs.

Antibiotics Concentrations ($\mu\text{g/ml}$) and culture response

Ampicillin	0	10	15	20	25	30	35	40	45	50
	+	+	+	+	+	+	+	+	+	+
Chloramphen.	0	1	2	4	6	8	10	15		
	++	++	++	+	+	+	+	+		
Kanamycin	0	10	15	20	25	30	35	40	45	50
	+	-	-	-	-	-	-	-	-	-
Streptomycin	0	25	50	75	100	125	150	175	200	
	+	+	+	+	(+)	-	-	-	-	
Tetracyclin	0	1	2	4	6	8	10	15		
	+	+	+	+	+	(+)	(+)	(+)		

+, Turbid; ++, Very Turbid; (+), Low Turbidity; chloramphen., chloramphenicol.

TABLE 3.1: ANTIBIOTIC RESISTANCE STUDIES FOR *R. palustris* W817

Metabolic Activity

R. palustris is characterized by both photoautotrophic and photoheterotrophic growth. Photoautotrophic growth occurs with hydrogen, sulfide and thiosulphate as electron donors in the presence of a small amount of yeast extract. However, for photoheterotrophic growth various organic compounds serve as substrates. Heterotrophic growth occurs in the dark under microaerobic to aerobic conditions and also under anaerobic conditions with some substrates (Truper and Imhoff 1989). This heterotrophic phenomenon of growth was studied for WS17 and ATCC 17001 employing several aliphatic and aromatic compounds in two different techniques, Biolog testing and auxanography as described in the materials and methods section.

1. Biolog GN ID Plates.

Utilization of organic compounds of various groups including simple sugars, alcohols and acids was studied with Biolog GN ID (Gram negative identification) Plates. These Biolog microtitre plates are designed on the principle of redox chemistry and the use of a colorimetric determination for assessing the metabolic activity of the inoculated organism. Metabolic fingerprints of WS17 and ATCC 17001 cultures under aerobic conditions in the presence or absence of light are presented in Table 3.2.

The results show that both the organisms have almost the same metabolic abilities with minor differences. In the absence of light WS17 showed positive reactions for itaconic

NO.	COMPOUND	WS17	ATCC17001
1.	Tween 40	L, D	L, D
2.	Tween 80	L, D	L, D
3.	L-arabinose	-, (D)	-, (D)
4.	Methyl pyruvate	L, D	-, -
5.	Mono-methyl succinate	L, D	L, D
6.	β -hydroxy butyric acid	L, D	L, D
7.	γ -hydroxy butyric acid	L, -	-, -
8.	Itaconic acid	L, D	-, -
9.	α -keto glutaric acid	L, D	-, -
10.	Propionic acid	L, D	L, D
11.	Sebacic acid	L, D	L, D
12.	Succinic acid	L, D	L, D
13.	Succinamic acid	L, D	L, D

L, +ve in presence of light; D, +ve in dark; (), weakly positive; -, negative.

Note : Results for all other substrates of Biolog plates were negative.

**TABLE 3.2. SUBSTRATE UTILIZATION BY WS17 AND ATCC17001
(BIOLOG TEST POSITIVE RESULTS)**

acid and α -ketoglutaric acid. Positive reactions were evident in the presence of light but only after incubation for 40 hours.

One reason for the difference between light and dark results may be the different temperature of incubation used, which was 26°C in the presence of light and 30°C in the dark. Under dark conditions a weak positive reaction was given by both the organisms for L-arabinose, however the reaction was negative in the presence of light. Another compound, Γ -hydroxy butyric acid, was used only by WS17 and only in the presence of light. Similarly, methyl pyruvate was metabolized only by WS17, but under both the conditions. Thus WS17 had somewhat different metabolic abilities in the presence and in the absence of light, when oxygen was available. ATCC 17001 gave positive reactions to the same substrates under both the conditions. Although WS17 had a wider substrate range than ATCC 17001, as indicated in Table 3.2, similarities in metabolic abilities, as tested by Biolog GN ID plates, was additional proof for the identification of WS17 as a strain of *R. palustris*.

2. Auxanography Technique.

Although, Biolog tests included a wide range of organic compounds, a different technique was necessary to study the utilization of aromatic substrates (custom designed Biolog plates have only recently become available). Also, Biolog microplates are not appropriate for the analysis of substrate

utilization profiles under anaerobic conditions. The redox chemistry of these tests depends on the presence of a fully induced electron transport system in the test strain and requires the presence of oxygen. Hence the Biolog GN ID plates were used to study the metabolic capabilities of WS17 and ATCC 17001 only under aerobic conditions in the presence and absence of light. For investigations of the substrate range under anaerobic conditions, an auxanographic technique was employed. Auxanography is a rapid and simple technique. It provides a range of concentrations of a substrate in a single plate for an organism to grow. Various substrates, including simple organics and halogenated as well as non-halogenated aromatic compounds were tested (Table 3.3). The substrates applied (one per plate) as solids, diffused through the medium and formed a concentration gradient in the plate along the line of diffusion. Growth of WS17 was observed either as an arc of bacterial biomass, surrounding a clear zone around the added substrate, or as uniform lawn all over the plate. The observations are presented in Table 3.3.

A picture of a typical auxanographic plate is presented in Figure 3.7. It shows an arc of bacterial growth surrounding benzoate, however, the negative control plate with no carbon source shows absence of growth. The substrates which produced an arc at a distance from the point of application (e.g. 3-chlorobenzoate), are probably toxic at higher concentration but support growth at low concentration. Those producing an

NO.	SUBSTRATES	OBSERVATIONS
1.	Acetate	Arc of heavy growth, very close to the substrate.
2.	Aspartate	No arc was seen. Low density lawn of growth was present.
3.	Citrate	
4.	Glucose	No arc seen. High density lawn of growth was present.
5.	Propanol	
6.	Sorbitol	
7.	Succinate	Same as that for aspartate.
8.	Tartrate.	
9.	Benzoate	Arc of heavy growth, close to the substrate.
10.	3-Bromobenzoate	No arc was seen. Low density lawn of growth was present.
11.	3-Chlorobenzoate	Arc of low density growth, away from the substrate.
12.	4-Chlorobenzoate	No arc was seen. Low density lawn of growth was present.
13.	2-Chlorobenzoate	
14.	2,4-Dichlorobenzoate	
15.	3,4-Dichlorobenzoate	
16.	2,5-Dichlorobenzoate	
17.	3,5-Dichlorobenzoate	
18.	Cyclohexane carboxylate	Arc of heavy growth, close to the substrate.
19.	Pimelate	
20.	4-Chlorophenol	Neither arc nor lawn were seen.

TABLE 3.3 : OBSERVATIONS OF AUXANOGRAPHIC PLATES

**FIGURE 3.7 : PRESENTATION OF SUBSTRATE UTILIZATION USING
AUXANOGRAPHIC PLATES**

Either no carbon (left) or benzoic acid (Ba, right) was added to the agar plates in the top centre. The plates were then incubated in anaerobic jars for up to two weeks. Growth is evident by an arc of pigmentation on the medium surface.

arc of growth very close to the substrate application point (e.g. sodium acetate) or supporting uniform lawn of high density all over the plate (e.g. glucose and propanol), may be good growth substrates at all concentrations, attained in the plate. The yield of cells with some substrates was low (e.g. aspartate and citrate), which may be due to application of insufficient amount of the compound or because of poor diffusion through the medium due to insoluble nature of the compound or they may be poor substrates for phototrophic growth of WS17. The ability of a compound to serve as a growth substrate or to be a better substrate than the other depends on its redox potential and its position in the electron tower (availability of potential energy with the compound). To provide energy to a cell the compound should be more reduced i.e. should bear more potential energy. e.g. Glucose is the reduced substrate in the pair at the top of electron tower and has the maximum amount of potential energy. Hence it was expected to and in fact has supported a heavy bacterial growth on agar plates.

Auxanography results for WS17 with aromatic compounds indicated that non-halogenated compounds supported better growth than the halogenated compounds. The results obtained for simple organic compounds when compared with the list of photosynthetic electron donors and carbon sources presented in the literature for *R. palustris* indicated that WS17 could be classified as a strain of *R. palustris* (Truper and Imhoff

1989). The ability of WS17 to metabolize benzoate in liquid as well as solid medium was perhaps the most important property supporting this identification, as *R. palustris* is the only species of non-sulfur, phototrophic bacterium known to be capable of benzoate degradation (Truper and Imhoff 1989; Pfennig and Truper 1989).

Plasmid Screening

For many aerobic microorganisms, the property of aromatic degradation is a plasmid borne character (Chatfield and Williams 1986; Dagley 1986; Fulthorpe and Wyndham 1989; Wyndham and Straus 1988a and Wyndham et al. 1988b). However, only one report has attributed an anaerobic metabolic phenotype for a aromatic compound to the presence of a plasmid. That is the benzoate degrading *Alcaligenes xylosoxidans* sub species *denitrificans* strain PN-1 with its indigenous plasmid pCBI (Blake and Hegeman 1987). Information is not available regarding the association of metabolic activities and plasmids in other anaerobes. A single report available till now on genetics of anaerobic phototrophic degradation of aromatics in *R. palustris* deals with 4-hydroxybenzoate and indicates that the degradation gene is chromosomal (Dispensa et al. 1992). With the anaerobic photosynthetic bacterium WS17, an attempt was made to find out whether anaerobic chlorobenzoate degradation was a genomic or a plasmid borne character. For this purpose several standard procedures of plasmid isolation were performed. An isolate of

R. palustris strain Rps5 (described in Chapter 2) was used as a positive control. Rps5 showed the presence of plasmids for all protocols tried, but there was no visible plasmid extracted from strain WS17. Other strains of *R. palustris*, CGA001, CGA033, CGA047, non-degraders of 3-chlorobenzoate (Harwood and Gibson 1988) and ATCC17001 were also tested for the presence of plasmid using a quick method of Krieg and Melton (promega technical bulletin). At least one plasmid was seen in all of these organisms (Figure 3.8). These results, showing an absence of plasmid in the active dehalogenating organism WS17 (as determined by various protocols) and the presence of plasmid in 3-chlorobenzoate non-degrading strains, indicated that the halobenzoate degradation property was probably not a plasmid borne character.

Despite the lack of correlation between plasmid content and phenotype, the indigenous plasmid of the ATCC 17001 strain was examined as a potential cloning vector. The size of the plasmid for 3-chlorobenzoate non-degrading strains was determined through restriction digestion of an ATCC 17001 plasmid preparation with *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III. The restriction pattern with these enzymes is presented in Figure 3.9. It shows only one restriction site each for *Bam*HI and *Eco*RI which linearized the plasmid. For *Bgl*II and *Hind*III the plasmid bears a minimum of four and two sites respectively. The single band in *Bam*HI and *Eco*RI digests at a distance of 2.3 cm from the point of origin on the gel coincides with the

FIGURE 3.8 : PLASMIDS IN VARIOUS STRAINS OF *R. palustris*

Lane #: 1, Lambda DNA: *Hind*III digest
2, pBRC60 (large plasmid control)
3, *R. palustris* CGA001
4, CGA033
5, CGA047
6, Rps-5
7, WS17
8, ATCC17001

1 2 3 4 5 6 7 8

FIGURE 3.9 : RESTRICTION PATTERN FOR *R. palustris* ATCC17001
CRYPTIC PLASMID

Lane # : 1, Lambda DNA *Hind*III digest
2, No sample
3, *Bam*HI
4, *Bgl*I
5, *Eco*RI
6, *Hind*III

1 2 3 4 5 6

position of a band for the second fragment of *Hind*III digested lambda phage DNA. Calculations based on the fragment sizes given in the literature for this fragment suggest that the plasmid is approximately 9.3 kb in size (Sambrook et al. 1989).

These observations lead to the conclusion that the isolate WS17 obtained from an acclimatized mixed culture is at least partially responsible for 3-chlorobenzoate degradation. It is the first reported photosynthetic bacterium involved in the degradation of halogenated aromatics. Characterization of WS17 conducted using several criteria identify this isolate as a strain of *R. palustris* similar to the type strain ATCC 17001.

CHAPTER 4

UPTAKE AND MINERALIZATION OF 3-CHLOROBENZOATE BY *R. palustris* WS17 AND REQUIREMENTS FOR ITS METABOLISM

INTRODUCTION

Anoxygenic phototrophic bacteria in the environment either grow as photoautotrophs using CO₂, or as photoheterotrophs using organic compounds as sources of carbon. This ability of photoheterotrophic growth is of great significance considering the increasing number of organic pollutants being added to the environment. Several such aromatic and non-aromatic organic compounds serve as growth substrates for the members of the family *Rhodospirillaceae* (Berry et al. 1987; Evans 1977; Sleat and Robinson 1984; Young 1984). *R. palustris* is reported to be the most versatile anaerobic phototroph, using a number of substituted and unsubstituted aromatic compounds (Harwood and Gibson 1988). The organism has definite prerequisites for utilization of these compounds (Harwood and Gibson 1988; Hutber and Ribbons 1983). The ability of the *R. palustris* isolate WS17 to metabolize 3-chlorobenzoate was confirmed through mineralization studies using [U-¹⁴C]-3-chlorobenzoate as presented in this chapter. The requisites of 3-chlorobenzoate metabolism in WS17 were also determined by studying various growth parameters.

MATERIALS AND METHODS

MINERALIZATION OF ^{14}C -3-CHLOROBENZOATE

Mineralization of $[\text{U-}^{14}\text{C}]$ -3-Chlorobenzoate by WS17 was determined by using medium P with 1 mM non-radioactive benzoate and 0.5 mM of uniformly ring labelled ^{14}C -3-chlorobenzoate (Sigma Radiochemicals, final activity 2 MBq/mmol). Cells were grown in glass tubes (15 mm x 125 mm) capped with rubber serum stoppers and containing 13.5 ml of medium P. Analysis was done on replicate tubes (n, number of replicates was 3) at regular intervals as follows. A sample of 1 ml of the culture was removed from the tube with a syringe and filtered through a 0.2 μm pore size polycarbonate Nuclepore filter. The filter was washed twice with 3 ml of 0.5 mM 3-chlorobenzoate solution and was dried. The dried filter was dissolved in 0.5 ml of ethanolamine in a glass scintillation vial prior to addition of 10 ml of scintillation fluid (Amersham ACS). The radioactivity was counted in a LKB Beta-Rak scintillation counter. The filtrate from the sample was used for HPLC analysis. Into the remaining liquid from the culture tube, 0.2 ml of concentrated KOH solution was injected, mixed thoroughly and the tube was allowed to stand for 1 to 1.5 hours at room temperature. At the end of this period another 1 ml sample for determination of total dissolved ^{14}C -carbon was taken from the tube. It was centrifuged and the radioactivity of the supernatant was

determined by counting 1 ml in 10 ml of ACS scintillation fluid.

The remaining culture volume (11.5 ml) was transferred to an Erlenmeyer flask for double vial respirometry. A scintillation vial containing 1 ml of ethanolamine was placed in the Erlenmeyer, then 2 or 3 drops of concentrated HCl were added to the culture volume in the flask and it was immediately closed tight with a rubber stopper. The flask was allowed to stand overnight with intermittent swirling. The scintillation vial was then removed and the ethanolamine was supplemented with 10 ml of ACS scintillation fluid and 3 ml of methanol. The radioactivity of trapped $^{14}\text{CO}_2$ was determined as described above. Finally, a 1 ml sample was taken from the acidified culture volume and dissolved organic- ^{14}C was measured as described above. All counts were calculated on a per culture volume (13.5 ml) basis.

THE REQUISITES FOR 3-CHLOROBENZOATE METABOLISM IN WS17

To determine the optimum conditions for 3-chlorobenzoate degradation by WS17, parameters like oxygen, light, pH, temperature and the requirement for cosubstrates were considered. The experiments were conducted using batch culture systems. Medium P was used in all of these experiments. Benzoate and 3-chlorobenzoate concentrations employed during each experiment were as shown in the graphical presentation of data. Growth and substrate disappearance were monitored as

described in Chapter 2.

The Requirements Of Oxygen, Light And A Cosubstrate.

To investigate the requirements of oxygen, light and a cosubstrate for 3-chlorobenzoate metabolism in WS17, aerobic light, aerobic dark and anaerobic light conditions were chosen. The experiment was conducted in two sets using a batch culture system (as described in Chapter 2). One set contained medium P with 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate and was inoculated with the mid exponential growth phase culture of WS17, growing on yeast extract medium (ATCC 112). The second set contained 0.5 mM 3-chlorobenzoate without a cosubstrate.

The Role Of Benzoate.

To establish the role of benzoate for 3-chlorobenzoate degradation in WS17, cultures were prepared by preincubation with benzoate and succinate in two separate bottles. WS17 cells preincubated with benzoate were considered induced for aromatic metabolism, while succinate pre-exposed cells were considered non-induced. These induced and non-induced cells were used to inoculate medium P containing 3-chlorobenzoate with and without benzoate.

The Range Of Cosubstrates.

To identify the range of possible cosubstrates for 3-chlorobenzoate metabolism in WS17, 1 mM each of cyclohexane carboxylate, pimelic acid, sodium acetate, sodium benzoate and sodium succinate were added separately to the medium P

containing 0.5 mM 3-chlorobenzoate. To prepare an inoculum, cells were harvested from a logarithmically growing culture of WS17 on yeast extract medium (ATCC medium 112), by centrifugation at 7000 rpm for 20 minutes and washing with TEA-PO₄ buffer (pH 7.2). Substrate disappearance was noted in each case and the maximum rates of 3-chlorobenzoate utilization attained by WS17 were compared to determine the most suitable cosubstrate.

The Requirements Of Optimum pH And Temperature.

The optimum pH necessary for isolate WS17 to acquire the maximum rate of chlorobenzoate metabolism was estimated using medium P containing 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate with variable pH values ranging from 6.05 to 8.55. Incubation was done at a constant temperature of 26°C in all cases. OD₆₆₀ of WS17 culture at 0 hour in all cultures was about 0.2. Optimum temperature for 3-chlorobenzoate degradation in WS17 was estimated using a constant pH medium P (pH 7.2) and incubating cultures at temperatures ranging from 20°C to 44°C. An exponential growth phase culture of WS17 growing on medium P was used as an inoculum in all the cases. At 0 hour of the experiment OD₆₆₀ was in the range of 0.066 to 0.07.

RESULTS AND DISCUSSION

UPTAKE AND MINERALIZATION OF ^{14}C -3-CHLOROBENZOATE

Metabolism of 3-chlorobenzoate by isolate WS17 was confirmed by supplementing the medium with [ring- ^{14}C]-3-chlorobenzoate and measuring the ^{14}C -label in products. Figure 4.1 shows the disappearance of ^{14}C -3-chlorobenzoate from culture medium and the accumulation of ^{14}C -label in cell biomass and $^{14}\text{CO}_2$. The data points presented are average values for the replicate analyses ($n=3$) with maximum of 3% standard deviation. Figure 4.1 shows that the rate of metabolism of 3-chlorobenzoate increased to a maximum of 0.045 mM per day between 4 and 8 days of incubation, and then slowed. The incorporation of label into biomass increased rapidly in the first 6 days of culture, followed by a marginal increase between 6 and 18 days. This indicated that the uptake and mineralization of chlorobenzoate by WS17 cells was correlated with the disappearance of substrate from the medium. Figure 4.1 shows that the label found in the CO_2 fraction reached a maximum after 6 days and then remained constant. This conversion of the substrate into CO_2 was equivalent to 20% of the available label at the stationary phase for several replicate cultures. The maximum rate of 3-chlorobenzoate degradation by WS17 in this experiment (0.045 mM per day) was not as rapid as observed in cultures

**FIGURE 4.1: METABOLISM OF [U- ^{14}C]-3-CHLOROBENZOATE
BY *R. palustris* WS17.**

A legend to the symbols used for ^{14}C -label detected in the original substrate, cell biomass and carbon dioxide is given in the figure (n=3).

transferred repeatedly in mid-exponential phase. In the latter, rates of 3-chlorobenzoate degradation of 0.15 mM per day were achieved. This difference in degradation rates was most probably due to the difference in status of the inoculum. An inoculum of exponential growth-phase cells was found to give higher rates of substrate degradation than a stationary phase inoculum.

The results of ^{14}C -3-chlorobenzoate uptake studies presented in figure 4.1 indicate that approximately 60% of the ring carbon atoms of 3-chlorobenzoate become incorporated into cellular biomass, while some 20% were mineralized to $^{14}\text{CO}_2$. The expected appearance of radioactivity in dissolved metabolite fractions was not observed when these were subjected to HPLC fractionation and counting. The balance of the label (20%) remained in the form of 3-chlorobenzoate in this experiment. If experiments were extended or if additional cosubstrate (benzoate) was added, 3-chlorobenzoate was degraded beyond the detection limit of the HPLC analysis (about 10 μM concentration). Figure 4.1 shows significantly higher radioactivity in cell biomass than in $^{14}\text{CO}_2$, and an apparent decline in the rate of formation of $^{14}\text{CO}_2$ after 6 days while incorporation of label into biomass continued to increase up to 15 days. This may be explained by postulating that $^{14}\text{CO}_2$ produced from ^{14}C -3-chlorobenzoate was simultaneously fixed by the growing cells. A similar reduction in CO_2 yield from 0.7 mole to 0.5 mole per mole of succinate

was reported by Ormerod (1956). That active CO₂ fixation in *R. palustris* was occurring is supported by the observation of carboxysomes in *R. palustris* WS17 growing on benzoate and 3-chlorobenzoate (Figure 3.5A).

The observations on ¹⁴C-3-chlorobenzoate mineralization presented in Figure 4.1 suggest that the chlorinated substrate is capable of serving as a significant source of carbon for biosynthesis.

THE REQUISITES OF 3-CHLOROBENZOATE METABOLISM IN WS17

The photosynthetic bacterium *R. palustris* is reported to degrade several aromatic compounds under specific environmental conditions (Harwood and Gibson 1988; Hutber and Ribbons 1983). A set of such ideal conditions required for 3-chlorobenzoate metabolism in isolate WS17 was estimated through the various experiments on oxygen availability, pH and temperature optima, and the requirement for co-substrates, as reported in the following section.

The results presented for benzoate and 3-chlorobenzoate degradation based on HPLC analyses are variable within maximum 10% of the reported value due to variations during separate machine operations. This instrumental error was considered while interpreting and comparing results. The data points presented in figures are average values in case of replicate analyses.

Requirements Of Oxygen, Light And A Cosubstrate

To study the requirements of these parameters for 3-chlorobenzoate metabolism by WS17, aerobic light, aerobic dark and anaerobic light conditions were chosen, in the presence or absence of benzoate. Figure 4.2A shows that 3-chlorobenzoate was metabolized by *R. palustris* WS17 with benzoate as a cosubstrate only under anaerobic conditions, in the presence of light. There was no degradation of 3-chlorobenzoate under aerobic conditions either in the presence or the absence of light.

Similar experiments were conducted without adding benzoate to the medium. The results from these experiments (Figure 4.2B) show that no degradation of 3-chlorobenzoate occurred under any of these conditions. The results presented in Figure 4.2 A and B indicate the necessity for benzoate as a co-substrate for metabolism of 3-chlorobenzoate, and also the necessity for light and anaerobic conditions. This requirement for benzoate as a cosubstrate in order to achieve complete degradation of the halogenated compound indicated that 3-chlorobenzoate alone was unable to act as an efficient growth substrate for WS17.

The Role Of Benzoate

An absolute need for benzoate in the medium to support the metabolism of 3-chlorobenzoate suggests that 3-chlorobenzoate alone is a poor growth substrate and it may be that it is incapable of inducing the metabolic enzymes of the

FIGURE 4.2 A and B**A: METABOLISM OF 3-CHLOROBENZOATE UNDER VARIOUS CONDITIONS
IN THE PRESENCE OF BENZOATE.**

A legend to the symbols used to indicate environmental conditions is given in the figure (n=2).

**B: METABOLISM OF 3-CHLOROBENZOATE UNDER VARIOUS CONDITIONS
IN THE ABSENCE OF BENZOATE.**

A legend to the symbols used to indicate environmental conditions is given in the figure (n=2).

A **B**

degradative pathway. Most probably metabolism requires benzoate to act as an inducer. The role of benzoate as an inducer was examined by following 3-chlorobenzoate metabolism by induced and non-induced cells.

In the case of induced cells (Figure 4.3A) 3-chlorobenzoate and benzoate degradation started immediately after inoculation. In 4 days nearly 0.35 mM of chlorobenzoate was degraded simultaneously with benzoate and was completely exhausted in 8 days, showing an overall rate of 0.06 mM per day. The maximum rate of chlorobenzoate metabolism achieved on the third and fourth days was 0.14 mM per day.

In the case of non-induced cells (Figure 4.3B), although benzoate degradation started immediately after inoculation, chlorobenzoate was degraded only after benzoate was completely exhausted from the medium, on the fifth day. Nearly 0.26 mM of chlorobenzoate was utilized by cells by the sixth day, with the maximum rate of utilization occurring on day 5 at 0.13 mM per day. From the seventh day onwards the degradation was very slow and was complete by 20th day with an overall rate of 0.025 mM per day. This slow metabolic activity of non-induced cells and the initial lag in 3-chlorobenzoate metabolism, in contrast to its rapid degradation by induced cells of WS17 without any lag, clearly indicated that benzoate acts as an inducer. This point is discussed further below.

Results from the experiments conducted with induced and non-induced cells using medium P lacking benzoate

FIGURE 4.3 A and B**A: BENZOATE AND 3-CHLOROBENZOATE METABOLISM BY
INDUCED WS17 WITH YEAST EXTRACT.**

A legend to the symbols used to indicate substrate conditions is given in the figure (n=2).

**B: BENZOATE AND 3-CHLOROBENZOATE METABOLISM BY
NON-INDUCED WS17 WITH YEAST EXTRACT.**

A legend to the symbols used to indicate substrate conditions is given in the figure (n=2).

FIGURE 4.3 C and D

**C: BENZOATE AND 3-CHLOROBENZOATE METABOLISM BY
INDUCED WS17 IN ABSENCE OF YEAST EXTRACT.**

A legend to the symbols used to indicate substrate conditions is given in the figure (n=2).

**D: GROWTH PATTERN OF INDUCED WS17 IN PRESENCE
AND IN ABSENCE OF YEAST EXTRACT.**

A legend to the symbols used to indicate substrate conditions is given in the figure (n=2).

2

PM-1 3 1/2"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

No	Initial Conc. (mM)		All Ba used on	3CBa metabolized (mM) at the end of				Max. OD660
	Ba	3CBa		Day	Day 4	Day 11	Day 17	Day 23
1.	0.25	0.5	4 (2)	0.1	0.18	0.3	0.31	0.712
2.	0.5	0.5	5 (2)	0.28	0.35	0.353	ND	0.867
3.	1.0	0.5	5 (3)	0.33	0.5 [9]	ND	ND	1.377
4.	2.0	1.0	9 (5)	0.325	0.825	0.895	1.0	1.599
5.	4.0	2.0	19 (7)	0.21	0.52	0.65	1.44	2.070
6.	6.0	3.0	23 (20)	0	0	0	0.34	2.263
7.	2.5	2.5	19 (11)	0.3	0.45	0.65	0.9	1.657
8.	0.5	1	4 (3)	0.27	0.7	0.71	ND	0.966

(), The day benzoate was almost exhausted; [], The day chlorobenzoate exhausted; ND, Analysis not done.

TABLE 4.1 : THE EFFECT OF BENZOATE CONCENTRATION ON METABOLISM OF 3-CHLOROBENZOATE BY *R.palustris* WS17.

(Figure 4.3A and B) showed the disappearance of a small amount (0.05 mM) of chlorobenzoate by induced cells in the first 8 days with no further decrease in its concentration. However, non-induced cells showed no degradation. The slight degradation activity of induced cells during the initial period just after inoculation, in the absence of benzoate, must be due to the persistence of some level of the benzoate reductive metabolic system in these cells after benzoate was removed. These observations also support the role of benzoate as an inducer for 3-chloro-benzoate metabolism in WS17.

Benzoate undergoes anaerobic metabolism through the reductive ring fission pathway (Dutton and Evans 1968b and 1969; Guyer and Hegeman 1969; Williams and Evans 1975). It is likely that 3-chlorobenzoate is dehalogenated to benzoate and its further metabolism proceeds via the same set of enzymes as for benzoate. 3-chlorobenzoate alone is incapable of inducing this pathway in WS17 but once it is induced by the co-substrate, then metabolism proceeds reasonably efficiently with the halogenated substrate serving both as a source of carbon and a reductant for phototrophic growth. This hypothesis is supported by the observations on ^{14}C -3-chlorobenzoate assimilation (Figure 4.1), where in the presence of benzoate a significant amount of 3-chlorobenzoate was taken up by WS17 cells, incorporated into cell biomass as well as mineralized to $^{14}\text{CO}_2$.

The Effect Of Benzoate Concentration.

As benzoate plays a significant role in 3-chlorobenzoate metabolism, a further set of experiments was conducted to determine the effect of benzoate concentration on the rate of 3-chlorobenzoate metabolism. For this purpose an initial experiment was conducted using medium P containing 0.5 mM of 3-chlorobenzoate, with benzoate in the concentration range 0.25 mM to 1.0 mM (Table 4.1). In all these cases benzoate was exhausted by day 4 with simultaneous degradation of 3-chlorobenzoate. Except for the medium containing 1 mM of benzoate, this was followed by a very slow and incomplete 3-chlorobenzoate degradation. Comparisons based on the overall rates of 3-chlorobenzoate metabolism for these four days showed an increase in the rate with the increase in benzoate concentration. The medium with 1.0 mM benzoate presented the maximum rate and complete degradation. The ratio for cosubstrate:substrate in this medium was 2:1. Maintaining this ratio the concentration of benzoate was increased up to 6.0 mM in subsequent experiments. Results presented in Table 4.1 for higher benzoate concentrations indicate that benzoate remains in the medium for a longer time and the 3-chlorobenzoate degradation rate decreases with the increase in concentrations. Further changes in the ratio to 1:1 or 1:2 with reduced concentrations did not help to enhance the metabolic rate. The results from Table 4.1 for the maximum OD₆₆₀ attained by cultures growing with various benzoate

concentrations show that a low density was attained by WS17 cultures at lower concentrations of benzoate. This may be one of the reasons for the lower rates of metabolism of 3-chlorobenzoate at these lower cosubstrate concentrations. At higher concentrations, although benzoate supports high culture density, it remains in the medium for a longer period and most probably competes with chlorobenzoate for the active sites of the enzymes of the reductive ring fission pathway. This further supports the hypothesis that benzoate and 3-chlorobenzoate are metabolized by identical pathways. The effect of culture density on the metabolic rate was investigated by substituting yeast extract (a nutrient source present in the medium supporting high culture density) from the medium P with the *R. palustris* growth factors *p*-aminobenzoic acid and biotin as described in the following section.

The Effect Of Culture Density.

Investigations were carried out to identify the effect of culture density on the rate of benzoate and 3-chlorobenzoate metabolism in WS17. High culture densities in any medium were obtained by supplementing the medium with yeast extract which acts as a source of several nutrients and growth factors for *R. palustris* (Brock and Madigan 1988). Therefore to understand the effect of culture density on 3-chlorobenzoate metabolism, medium P with and without yeast extract was used. The medium without yeast extract was supplemented with 0.15 mM of *p*-aminobenzoic acid and 0.15 mM biotin. A benzoate induced

culture of WS17 was used as an inoculum.

The results (Figure 4.3C) showed very slow and incomplete degradation of benzoate as well as chlorobenzoate in the absence of yeast extract. Only 0.37 mM benzoate and 0.175 mM 3-chlorobenzoate were metabolized within the first 7 days, followed by very slow metabolism of only benzoate up to the 13th day. No further degradation was seen from the 8th day onwards for 3-chlorobenzoate and from the 14th day onwards for benzoate. This may be because the culture attained a very low density ($OD_{660} = 0.516$) and reached stationary phase very early as depicted by growth curves based on the absorbance at 660 nm (Figure 4.3D). In contrast, for medium P with yeast extract the stationary phase was reached by the 8th day and the density of the culture was high ($OD_{660} = 1.782$). Benzoate from this medium was exhausted very rapidly by the 4th day and 3-chlorobenzoate was metabolized by the 8th day (Figure 4.3A).

These results suggest the inability of *p*-aminobenzoic acid and biotin to replace yeast extract as a source of growth factors. Yeast extract supplies more than these two essential vitamins and supports faster and more dense growth of WS17. These results combined with those for the effect of benzoate concentration suggest that the rate of substrate utilization depends on the density of the culture.

The Range Of Cosubstrates

The data from previous experiments shown in figures 4.3A and B suggest that the cosubstrate, benzoate, induces 3-

chlorobenzoate metabolism in WS17, which probably follows the reductive ring fission pathway. Therefore, the metabolites of the benzoate pathway may also act as cosubstrates. A few of these were tested for their ability to support 3-chlorobenzoate metabolism in WS17. The maximum rates of chlorobenzoate utilization attained by WS17 in the presence of each of these cosubstrates were compared to identify the most suitable compounds to be used as cosubstrates. The comparison showed that benzoate and cyclohexane carboxylate were equally effective as cosubstrates. Maximum rates of 3-chlorobenzoate utilization achieved in the presence of these two compounds were nearly equal (0.111 mM per day for benzoate and 0.106 mM per day for cyclohexane carboxylate).

Cyclohexane carboxylate is reported to induce benzoate utilization through the production of an acyl CoA synthetase (benzoyl CoA synthetase) which is active with benzoate (Hutber and Ribbons 1983; Blakley 1978). Therefore it was expected that this compound would also act as an efficient cosubstrate.

Another metabolite, pimelic acid, was found to be a less efficient cosubstrate, resulting in a lower rate (0.084 mM per day) of 3-chlorobenzoate metabolism. The ability of pimelic acid to induce the benzoate pathway was not examined here, and neither is it reported in the literature. However, since pimelic acid supported the metabolism of 3-chlorobenzoate at a reasonable rate it may be inducing the reductive ring fission pathway.

Acetate and succinate, intermediates of the citric acid cycle, were also found to support 3-chlorobenzoate metabolism, but the metabolic rates were quite low (0.046 mM per day for succinate and 0.022 mM per day for acetate). While succinate is reported to induce benzoyl CoA synthetase, the activity is found to be very low (Hutber and Ribbons 1983). However, this finding may explain the ability of succinate to act as a cosubstrate. Similarly acetate may also have an ability to produce benzoyl CoA synthetase as indicated by the results here. Although, information regarding the induction of benzoate utilization by acetate is not available in the literature acetate is reported as a source of reducing potential for reductive dehalogenation (Mohn and Tiedje 1990).

The results obtained here suggest that benzoate and cyclohexane carboxylate are equally efficient cosubstrates for 3-chlorobenzoate metabolism as higher rates of metabolism were achieved in their presence.

Optimum pH And Temperature For 3-Chlorobenzoate Metabolism

To ascertain the optimum pH for the maximum rate of 3-chlorobenzoate metabolism in WS17, disappearance of the substrate from medium P with the pH values 6.05, 6.5, 6.9, 7.15, 7.25, 7.65, 7.8 and 8.55 was determined. pH Variation over total period of incubation in any case was approximately ± 0.03 . Typical results for pH variation in medium P containing benzoate and 3-chlorobenzoate, and benzoate alone, are presented in Appendix-III. The maximum rate of substrate

utilization was calculated for each of these media as the maximum slope of tangent to the degradation curve. Figure 4.4 depicts the variation of these rates with the corresponding pH values. There was a low rate of degradation at pH 6.05 (0.055 mM per day) which increased steadily with the increase in pH and reached a maximum value of 0.118 mM per day at pH 7.25. Further increases in the pH reduced the rate, thus showing a clear optimum for 3-chlorobenzoate degradation. At pH 8.55 there was very little increase in optical density of the culture. Cells formed large flocks probably due to pH sensitivity of the culture at high pH values. The cell density was determined at the points during degradation used to calculate rates and this value of cell density was converted to protein concentration. Expressing substrate utilization rates using these values as mM per day per milligram of protein gave a maximum rate of utilization of 0.557 mM per day per mg of protein at the optimum of pH 7.25.

The optimum temperature required for WS17 to attain the maximum rate of 3-chlorobenzoate degradation was determined by growing the cultures of WS17 at 20°C, 26°C, 32°C, 37°C, 40°C and 44°C in medium P at the pH of 7.2. Variation in the temperature of incubation during the entire period of examination was approximately ± 0.5 degree. Figure 4.5 shows variation of 3-chlorobenzoate degradation rates (calculated in the same way as described earlier for optimum pH determination experiment) with a change in the temperature of incubation.

**FIGURE 4.4: OPTIMUM pH FOR 3-CHLOROBENZOATE METABOLISM
IN *R. palustris* WS17.**

3-Chlorobenzoate metabolism rate was calculated as the maximum slope of the substrate disappearance curve at different starting pH conditions (n=1). The variation of pH during the course of experiments is given in Appendix III. When these rates were calculated on a mM/day/mg protein basis, there was no difference in the optimum pH.

**FIGURE 4.5: OPTIMUM TEMPERATURE FOR 3-CHLOROBENZOATE
METABOLISM IN *R. palustris* WS17.**

3-Chlorobenzoate metabolism rate was calculated as the maximum slope of the substrate disappearance curve at different temperatures (n=1). When these rates were calculated on a mM/day/mg protein basis, there was no difference in the optimum temperature.

3-Chlorobenzoate was metabolized at a lower rate (0.054 mM per day; equivalent to 0.417 mM per day per mg protein) when the incubation temperature was low (20°C). An increase in temperature up to 37°C gradually increased the rate, which then dropped with a further increase in temperature to 40°C and beyond. The maximum value for the rate of 3-chlorobenzoate metabolism by WS17 (0.119 mM per day; equivalent to 0.902 mM per day per mg protein) was achieved at 37°C, and this may be considered as an optimum incubation temperature to obtain the maximum rate of 3-chlorobenzoate metabolism.

The pH of a medium and the temperature of incubation, control metabolic activities of bacterial cultures. The requirements of these parameters vary with the organism, e.g. acidophilic organisms grow best at acidic pH; psychrophilic organisms grow at low temperatures. However, mesophilic organisms usually grow best at around neutral pH and in the temperature range of 30-37°C. For the photosynthetic bacterium *R. palustris* ATCC 17001, optimal growth is reported to occur at 30-37°C and pH 6.9 with the range 5.5-8.5 (Imhoff and Truper 1989). WS17 being a strain of *R. palustris* it was expected to grow optimally with the pH and temperature in these reported ranges. These optimal values were further verified for 3-chlorobenzoate metabolism in WS17 and were found to vary marginally as compared to those reported for optimal growth.

CHAPTER 5
MECHANISM AND KINETICS OF 3-CHLOROBENZOATE METABOLISM
IN *R.palustris* WS17

INTRODUCTION

Microorganisms belonging to the groups like methanogens, denitrifiers and sulfate reducers are known to degrade various substituted and unsubstituted aromatics under anaerobic conditions (Horowitz et al. 1983; Schennen et al. 1985; Sharak Genthner et al. 1989a and b; Suflita et al. 1982; Tiedje et al. 1987 and Urbanek et al. 1989; Mohn and Tiedje 1992). Similarly several reports demonstrate the ability of phototrophs to metabolize simple aromatics (Dutton and Evans 1969, 1978; Evans and Fuchs 1988; Harwood and Gibson 1988; Hegeman 1967; Rahalkar et al. 1991). The ability of phototrophs to degrade halogen substituted aromatics was not explored until recently (Kamal and Wyndham 1990; Montgomery and Vogel 1992).

The isolated strain *R.palustris* WS17, belonging to the α -class within the family Proteobacteria (Woese 1987), metabolized 3-chlorobenzoate in the presence of cosubstrates. Efficient metabolism occurred only under anaerobic conditions in the presence of light. Various requirements of the organism for this process were studied (Chapter 3) and the results indicated the possibility of 3-chlorobenzoate being degraded by a reductive ring-fission pathway in a similar way to that

of benzoate. Most probably the initial step involves either formation of a coenzyme A (CoA) conjugate followed by dehalogenation of the conjugate to form benzoyl-CoA or direct dehalogenation of chlorobenzoate followed by formation of benzoyl-CoA conjugate which enters reductive ring fission pathway of benzoate metabolism.

The dechlorination of 3-chlorobenzoate by a sulfidogenic bacterium *Desulfomonas tiedjei* DCB-1 produced stoichiometric amounts of Cl^- ions and benzoate (Dolfing and Tiedje 1987; Dolfing 1990; Mohn and Tiedje 1990). With the anaerobic metabolism of 2-fluorobenzoate by a benzoate-degrading, denitrifying bacterium, fluoride was reported to be released (Schennen et al. 1985). Similarly, dehalogenation of several mono, di and tri halobenzoates was observed in a methanogenic consortium (Suflita et al. 1982). In addition to halobenzoates this dehalogenation phenomenon is reported for a variety of other compounds, including chlorophenols (Boyd et al. 1983; Boyd and Shelton 1984; Mikesell and Boyd 1985, 1986); the pesticides 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (Mikesell and Boyd 1985), and polychlorinated biphenyl commercial mixtures called Arochlors (Brown et al. 1987; Montgomery and Vogel 1992; Nies and Vogel 1990; Quensen et al. 1988, 1990; Ye et al. 1992). Whether a similar mechanism to that observed in *D. tiedjei* DCB-1, consisting of dehalogenation prior to ring fission, exists in *R. palustris* WS17 is explored in this chapter.

MATERIALS AND METHODS

The 3-Chlorobenzoate metabolic pathway in *R. palustris* WS17 was investigated with three different approaches:

- 1) Direct metabolite identification through Gas Chromatography Mass Spectroscopy (GCMS) and HPLC analyses.
- 2) Indirect identification by using intermediates to inhibit metabolism.
- 3) Enzymatic studies using cell free extracts.

GCMS STUDIES

To identify the intracellular and extracellular products of benzoate and 3-chlorobenzoate metabolism in WS17, samples were prepared for GCMS analyses as follows. Cultures were grown on medium P containing only benzoate (1.0 mM); benzoate (1.0 mM) and 3-chlorobenzoate (0.5 mM); or cyclohexane carboxylate (1.0 mM) and 3-chlorobenzoate (0.5 mM). In consideration of the hypothesis that 3-chlorobenzoate metabolism follows a similar route to that of benzoate, the medium P with cyclohexane carboxylate was used to try and avoid interference of benzoate in the identification of metabolites of 3-chlorobenzoate. Cells were harvested from medium P with benzoate alone during the phase of its rapid metabolism to obtain the intermediates of benzoate metabolism. Whereas, from the medium P containing a cosubstrate and 3-

chlorobenzoate, the cells were harvested for analysis of intracellular intermediates of 3-chlorobenzoate degradation, only after the cosubstrate benzoate or cyclohexane carboxylate were exhausted and 3-chlorobenzoate was being rapidly degraded. The cells were obtained from these three different media by centrifugation at 7000 rpm for 20 minutes. The culture supernatants were extracted with diethyl ether (BDH Inc., Canada) for extracellular products. However, for the intracellular products the cell pellets were washed twice with 10 mM TEA-PO₄ buffer (pH 7.2) and cells were lysed using lysozyme treatment as per the procedure described in Appendix-II. The lysates were then extracted with diethyl ether. These ether extracts were treated with trimethyl silane (TMS) and were subjected to GCMS analysis. The gas chromatograph (Hewett Packard 5890 series II) with a 30m J&W DB5 column was operated at 120°C for 2 min. then raised 10° per min. to 300°C, and linked to a mass spectrometer (Concept 2H, Kratos Analytical, Manchester, England) operated at 70 eV energy accelerating at 8KV.

HPLC ANALYSES FOR INTRACELLULAR INTERMEDIATES

For identification of the intracellular intermediates of 3-chlorobenzoate metabolism using HPLC analyses two procedures were tried. In the first procedure samples were collected at a single point during the logarithmic growth phase, whereas for the second procedure sampling was done several times at intervals throughout the growth profile.

For the first procedure WS17 cells actively growing on medium P with benzoate alone were harvested during its rapid metabolism (for intermediates of benzoate metabolism). Whereas, the cells from medium P with benzoate and 3-chlorobenzoate were harvested by centrifugation immediately after benzoate was exhausted and rapid 3-chlorobenzoate degradation was in progress (for metabolites of 3-chlorobenzoate degradation). The cells were washed twice with TEA-PO₄ buffer (pH 7.2) and then were lysed by sonication for 2-3 minutes at near freezing temperatures using a Vibra Cell sonicator. The instrument was run for 10 to 12 seconds at one time with a pulsating cycle at 20% output. Cell lysis was monitored by phase contrast microscopy. Proteins from the lysate were precipitated using TCA and the supernatants obtained after centrifugation were used in HPLC analysis for intracellular products of benzoate and 3-chlorobenzoate metabolism. A part of the protein free lysate was treated with 1.0 mM NaOH (pH maintained at 12.0 for 10 minutes at room temperature) to hydrolyse thioesters and HPLC analyses were conducted again.

For the second procedure WS17 cultures growing on medium P with benzoate or with benzoate and 3-chlorobenzoate were harvested at the same metabolic stages as described for the aforementioned procedure. The cells from medium containing benzoate alone were benzoate induced. However, those from benzoate and 3-chlorobenzoate containing medium were 3-

chlorobenzoate induced cells. The washed cells were used to inoculate TEA-PO₄ buffer supplemented with *p*-aminobenzoic acid and biotin (0.15 mM each) and 0.5 mM 3-chlorobenzoate as a culture medium in two different sets. During the culture growth on 3-chlorobenzoate sampling was done several times at intervals. The samples were prepared for HPLC analyses from the WS17 cell lysate through protein precipitation and thioester hydrolysis as described in the first procedure above. The same protocol was tried using temperature shock treatment as described by (Merkel et al. 1989) to recover early intermediates of the pathway. However, the attempt was not successful probably due to instability of intermediates to high temperatures employed during the procedure. (The protocol for this technique includes boiling of cells followed by immediate transfer on ice).

DEHALOGENATION

For dehalogenation studies WS17 was grown in a minimal medium (Dutton and Evans, 1969). The medium was supplemented with 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate as organic carbon and also with 0.5 gm L⁻¹ of yeast extract as a source of amino acids and growth factors. Two types of inoculum were prepared, one with a WS17 culture rapidly degrading benzoate from the minimal medium containing only benzoate (benzoate induced cells) and the second from that growing with benzoate and 3-chlorobenzoate where benzoate was exhausted and 3-chlorobenzoate was undergoing rapid metabolism (3-chloro-

benzoate induced cells). The cells were harvested, washed with TEA-PO₄ buffer (pH 7.2) and were used as an inoculum. The benzoate induced cells were used to inoculate minimal medium with only benzoate whereas the 3-chlorobenzoate induced cells were used as an inoculum for the medium with benzoate and 3-chlorobenzoate. Growth and substrate disappearance were monitored as described in Chapter 2. The chloride concentration was measured by the modified mercury (II) thiocyanate method using a NaCl standard with spectrophotometric technique (Florence and Farrar, 1971). For these chloride analyses, to a 500 μ l sample, 100 μ l Iron (III) reagent, 100 μ l Mercury (II) thiocyanate reagent and 300 μ l Milli-Q water were added and mixed thoroughly by vortexing. Absorbance of this mixture was measured at 460 nm on a Beckman model 26 UV/visible spectrophotometer.

INTERMEDIATE INHIBITION

A WS17 culture was allowed to grow on medium P with benzoate (0.71 mM) and 3-chlorobenzoate (0.34 mM) until the benzoate was completely exhausted. This culture was then divided equally into 4 aliquots and each was spiked with a compound which may be expected as an intermediate of the 3-chlorobenzoate reductive pathway. Benzoate (0.57 mM), cyclohexane carboxylate (0.5 mM) and pimelic acid (0.5 mM) were employed for the purpose and yeast extract (0.1%) was used as a negative control. The cultures were grown further under anaerobic conditions in the presence of light. Growth

and substrate removal were monitored as described earlier in Chapter 2.

ENZYME STUDIES

The Cell Free System.

The presence of benzoyl CoA synthetase/ligase and its specificity were examined using crude cell free extracts of WS17 from an actively growing culture in medium P with benzoate alone (benzoate induced) or with benzoate and 3-chlorobenzoate (3-chlorobenzoate induced). The cells were harvested from these media at the metabolic stages as described for earlier procedures. The cell free extracts were prepared by lysozyme treatment followed by sonication and pH and temperature shock (Appendix-II). The assay system consisted of 10 mM TEA-PO₄ buffer (pH 7.25), 1 ml; 0.45 mM (final concentration) benzoate; 0.25 mM CoA; 0.5 mM ATP and 2.5 mM MgCl₂. The final volume of the reaction mixture was 2.2 ml. For an assay of 3-chlorobenzoate reactivity, benzoate was replaced by 0.23 mM 3-chlorobenzoate. The reaction was started by adding 1.2 ml cell free extract, containing approximately 0.5 mg protein. A zero time sample was taken and following incubation at 20°C reaction subsamples were stopped by adding 220 µl of 50% TCA. The samples were then chilled on ice and centrifuged at 13000 rpm for 10 minutes. From the supernatant free acid (substrate) was removed by acidifying and extracting it with 2.5 ml diethyl ether. The aqueous phase was analyzed

by HPLC for the product (Benzoyl CoA). For confirmation, in a few cases, CoA thioester was hydrolysed to free acid by NaOH treatment and was reanalyzed by HPLC.

RESULTS AND DISCUSSION

Radioisotopic studies using ^{14}C -3-chlorobenzoate confirmed the ability of WS17 to degrade 3-chlorobenzoate with benzoate as a cosubstrate (Chapter 4). As shown in figure 4.1 the incorporation of label into cell biomass and $^{14}\text{CO}_2$ production by WS17 were measured, however no radioactive metabolites of 3-chlorobenzoate were observed with fractionation using HPLC analyses. Due to this inability to obtain radioactive intermediates, the mechanism of 3-chlorobenzoate metabolism in WS17 could not be revealed with radioisotopic studies. However, several results presented in previous chapters indicated the possibility that metabolism occurs via a reductive ring-fission pathway similar to benzoate. An attempt was therefore made to establish the mechanism of 3-chlorobenzoate metabolism in WS17 through identification of metabolites using various approaches.

WHOLE CELL STUDIES (HPLC AND GCMS ANALYSES)

Identification of intermediates of the 3-chlorobenzoate degradative pathway was attempted by extracting culture media and cells and analyzing the extracts by GCMS and HPLC. HPLC analysis was conducted for samples (supernatants from cell lysates treated with TCA alone and with TCA and NaOH) of a WS17 culture growing on medium P with benzoate and 3-chlorobenzoate to identify intracellular products of 3-

chlorobenzoate degradation (sampled at the time the benzoate was completely exhausted and processed as described in above). The results of this analysis showed the presence of 3-chlorobenzoate, however benzoate and benzoyl CoA were not observed either prior to or after NaOH treatment (Table 5.1, columns 4 and 5). The samples were expected to show the presence of these compounds if 3-chlorobenzoate was degraded by the benzoate pathway. The most probable reason for the absence of these compounds may be the limitations of HPLC analyses in terms of detection limit. The detection limit of the instrument with UV absorbance measurement in the aromatic ring absorbance range (254 nm) was 10 μ M for the chlorinated aromatics and benzoate. Rapid turnover of intermediates in the active metabolic state of the cell may prevent the accumulation of significant intermediate pools. Although intermediates of 3-chlorobenzoate degradation were not identified during this analysis, evidence was provided for the intracellular presence of 3-chlorobenzoate.

Samples of WS17 from medium P with benzoate alone, analysed by HPLC to identify intracellular products of benzoate degradation, did not show the presence of either benzoate or benzoyl CoA in TCA precipitated lysates. However, further treatment of the protein free lysate for thioester hydrolysis resulted in the appearance of a benzoate peak (Table 5.1, columns 2 and 3). This was indirect evidence

Treatment	Metabolites of Benzoate Degradation		Metabolites of 3CBa degradation	
	Peak R. T.	I.D.	Peak R. T.	I.D.
Lysate + TCA	7.6 min	N.I.	3.95 min	N.I.
	12.6min	N.I.	4.4 min	3CBa
Lysate + TCA + NaOH	1.8 min	N.I.	1.9 min	N.I.
	2.25 min	Benzoate	3.45 min	N.I.

TCA, Trichloroacetic acid; R.T., retention time; I.D., Identification; N.I., not identified; 3CBa, 3-chlorobenzoate.

Lysate + TCA treatment indicates analysis after protein precipitation.

Lysate + TCA + NaOH treatment indicates analysis after protein precipitation and thioester hydrolysis

Note : The retention times for peaks of benzoate and 3CBa standards on the day of analysis were 2.206 and 4.394 min respectively.

TABLE 5.1 : INTRACELLULAR PRODUCTS OF BENZOATE AND 3-

CHLOROBENZOATE DEGRADATION IN *R. palustris* WS17

(Whole Cell Studies : Single Sampling Procedure)

indicating that some form of thioester conjugate of benzoate, probably benzoyl CoA, was formed in these cultures while degrading benzoate. (Benzoyl CoA, being a thioester, undergoes hydrolysis with alkali treatment to form benzoic acid and for this reason benzoate was observed after the treatment of lysate with NaOH). These results support the published findings indicating conjugation of the intermediates of the aromatic ring reduction pathway to coenzyme A in *R. palustris* (Harwood and Gibson, 1986; Hutber and Ribbons, 1983). It also suggests that the mechanism of benzoate degradation in WS17 is similar to that described for other strains of *R. palustris* (Geissler et al. 1988).

Chromatograms of the samples treated for thioester hydrolysis (i.e. after NaOH treatment of protein free lysate) showed a peak at a retention time of about 1.8 minutes during both benzoate and 3-chlorobenzoate degradation. This compound could not be identified based on comparisons for retention times of standard solutions of several aromatic compounds and expected intermediates.

The intracellular presence of 3-chlorobenzoate was confirmed by GCMS analyses. The results presented in Table 5.2 show that 3-chlorobenzoate is one of the compounds identified by GCMS analyses from the pool of metabolites present inside the cell. However, most of the other intracellular products identified were intermediates of fatty acid metabolism.

A unique finding which has not been reported in the

literature was the appearance of trace amounts of hydroxylated metabolites of the aromatic substrates observed in the GCMS analysis results (Table 5.2). *Ortho*- and *meta*-hydroxybenzoate and trihydroxychlorobenzoate appeared as extracellular products of benzoate and 3-chlorobenzoate metabolism in WS17. Non-productive hydroxylation of chlorinated aromatic substrates is a common observation for many aerobic microorganisms (Hickey and Focht 1990) and it is thought to be a result of the spontaneous dehydration of oxygenated intermediates during aerobic ring-fission. This pathway cannot occur in the anaerobic cultures described here. The hydroxylated metabolites observed in WS17 extracts may be products of hydration of the aromatic ring, a reaction that has been shown to occur under anaerobic conditions in several bacteria (Scholten et al. 1991). Alternatively, the reason for hydroxylation may be that benzoate (and 3-chlorobenzoate) are hydroxylated prior to ring reduction in WS17. While uptake of the hydroxylated compound, *p*-hydroxybenzoate, is known for *R. palustris*, its dehydroxylation on entry to the cell is not known (Merkel et al. 1989). Instead two separate degradative pathways are suggested for these compounds, each conjugated to coenzyme A. One is used for benzoate and the other for *p*-hydroxy-benzoate (Harwood and Gibson 1988). In the results presented here no hydroxylated substrates were identified

INTRACELLULAR PRODUCTS :	
Medium P with only Benzoate	Perrhenic Acid (TMS Ester), Pentatriacontane, Tetracosane.
Medium P with Benzoate and 3-Chlorobenzoate	Butadioic Acid, 2-Butenedioic Acid, 3-CHLOROBENZOATE , 11-Cis Octadecanoic Acid, Hexadecanoic Acid.
EXTRACELLULAR PRODUCTS :	
Medium P with only Benzoate	Benzoate, 2-HYDROXY BENZOATE , 3-HYDROXY BENZOATE .
Medium P with Benzoate and 3-chlorobenzoate	3-Chlorobenzoate TRIHYDROXY CHLOROBENZOATE , Phthaleic Acid.
Medium P with Cyclohexane carboxylate and 3-chlorobenzoate	3-Chlorobenzoate, Cyclododecane, Cyclohexane carboxylate, TRIHYDROXY CHLOROBENZOATE .

**TABLE 5.2 : METABOLIC INTERMEDIATES OF *R.palustris* W817
GROWING ON VARIOUS MEDIA**

intracellularly, and the hydroxylated metabolites found in the culture medium were in very low concentrations. These observations, combined with the results of intermediate inhibition studies to follow, suggested the observed hydroxylated metabolites were probably dead-end or non-productive metabolic products.

Aside from 3-chlorobenzoate itself, no intermediates of the metabolic pathway were identified from intracellular extracts. Another protocol including several sampling times during the growth of the culture was employed. In these experiments, HPLC analysis of both benzoate and 3-chlorobenzoate induced WS17 cultures, growing on 3-chlorobenzoate in TEA-PO₄ buffer, identified benzoate as an intracellular product in all the samples analyzed after treatment for thioester hydrolysis (Table 5.3). This was an indirect evidence for formation of coenzyme-A thioester conjugate. The presence of benzoate (indicating presence of benzoyl CoA prior to thioester hydrolysis) in 0 hour samples was not expected, however, the observed peak was very small and such minute concentration can be accounted for the metabolism of residual substrates from inoculum culture. None of the samples showed the presence of 3-chlorobenzoate inside the cells. The results indicate that 3-chlorobenzoate is dehalogenated to benzoate, either directly, or possibly as a CoA thioester.

Sampling Time	Benzoate induced cells		3CBa induced cells	
	Peak R.T.	I.D.	Peak R.T.	I.D.
0 hr.	1.680*	Benzoate	2.51*	Benzoate
15 min	1.674	Benzoate	2.569	Benzoate
30 min	1.645	Benzoate	2.540	Benzoate
45 min	1.644	Benzoate	2.480	Benzoate
1 hr.	1.321	Benzoate	2.819	N.I.
1.5 hr.	1.610	Benzoate	2.51	Benzoate
20.5 hr.	1.821	N.I.	2.920	N.I.
22.5 hr.	1.811	N.I.	N.D.	N.D.
24 hr.	1.751	N.I.	N.D.	N.D.
26 hr.	Tail peak at 1.6	N.I.	N.D.	N.D.
46 hr.	Tail peak at 1.5	N.I.	N.D.	N.D.

Note : The analysis was done on cell lysates of 3CBa degrading cells after protein precipitation and thioester hydrolysis.

For benzoate induced samples HPLC analysis was done on 13/4/91 and 14/4/91. On these dates retention time for benzoate standard were in the range 1.323 to 1.601 and 2.438 to 2.611 min respectively. Analysis for 3CBa induced cells was done on 14/4/91 and 15/4/91.

N.D., Not done.; *, minor peak indicating minute concentrations. All other symbols are same as in Table 5.1.

TABLE 5.3 : INTRACELLULAR PRODUCTS OF 3-CHLOROBENZOATE

DEGRADATION IN *R. palustris* WS17

(Whole Cell Studies : Multiple Sampling Procedure)

DEHALOGENATION

The phenomenon of dehalogenation was further investigated using induced WS17 cultures. Figure 5.1 presents the observed metabolism of benzoate and 3-chlorobenzoate in three different media with the corresponding release of chloride ions. As seen from the figure, a WS17 culture growing with 1.0 mM benzoate and 0.5 mM 3-chlorobenzoate (culture A) released an increasing amount of chloride with the progress in metabolism of 3-chlorobenzoate. This shows a definite correlation between metabolism and dehalogenation of the substrate. Nearly 35 μg of chloride was found in culture supernatants following metabolism of 0.56 mM 3-chlorobenzoate and 1.01 mM benzoate. Chloride (13 μg) was also observed for the culture growing on 1.0 mM benzoate in the absence of 3-chlorobenzoate (culture B). Such results were expected as the incubation medium was not free of chloride ions. Contamination from the yeast extract is a likely source of chloride. Considering this amount as a background level, net chloride released by WS17 in utilizing 0.56 mM of 3-chlorobenzoate was 22 μg , equivalent to 0.6 mM. A culture with only 3-chlorobenzoate as a substrate failed to significantly metabolize the chlorinated substrate and resulted in no net increase in chloride in the supernatant.

These results from dehalogenation studies, combined with those for the identification of intracellular products

**FIGURE 5.1: BENZOATE AND 3-CHLOROBENZOATE METABOLISM
AND CHLORIDE RELEASE BY WS17.**

A legend to the symbols used to indicate substrate and chloride concentrations is given in the figure.

Ba, benzoate; **3CBa**, 3-chlorobenzoate.

through HPLC and GCMS analyses, indicate that the halogenated substrate, 3-chlorobenzoate, is taken up by the cells and is dehalogenated to benzoate. This step may occur in the form of a Coenzyme A conjugate, however no evidence for this was found. Further metabolism of benzoate did involve a thioester intermediate and most likely involved the known benzoyl-CoA reductive pathway. Benzoate metabolism in WS17 was found to proceed through formation of a thioester of benzoate (results of HPLC analyses for the samples processed by the first procedure above, Table 5.1) in a similar way to that for other strains of *R. palustris*. Intermediate inhibition was then used to further establish the pathway, as described below.

INTERMEDIATE INHIBITION

Metabolism of 3-chlorobenzoate was expected to proceed via reductive ring fission after its dehalogenation to benzoate or benzoyl-CoA. To examine this hypothesis the known intermediates of the benzoate pathway, cyclohexane carboxylate and pimelic acid, were examined as metabolic inhibitors. The effects of addition of these compounds on the utilization of benzoate and 3-chlorobenzoate by WS17 are presented in Figure 5.2. The results show that addition of 1.0 mM benzoate (Ba) during the rapid phase of 3-chlorobenzoate (3CBa) degradation caused a marginal reduction in the rate of 3-chlorobenzoate utilization for a short period. However, addition of

**FIGURE 5.2: INHIBITION OF 3-CHLOROBENZOATE METABOLISM
IN WS17 WITH THE ADDITION OF SUSPECTED INTERMEDIATES.**

A legend to the symbols used to indicate substrate concentrations in separate incubations with various intermediates is given in the graph. The vertical line indicates the time of addition of intermediates.

Ba, benzoate; **3CBa**, 3-chlorobenzoate.

cyclohexane carboxylate (CHC) and pimelic acid (PIM) to the incubation medium halted 3-chlorobenzoate metabolism for a day, which resumed again most probably after these compounds were exhausted. Disappearance of the intermediate compounds could not be determined as their absorption wavelength range is beyond the working range of the HPLC detector. This phenomenon of preferential utilization of an added intermediate can be attributed to the availability of active enzymes for incorporation of intermediates into the pathway, and the sudden build up of an intracellular pool of intermediates which effectively blocks the utilization of the original substrate. Compounds which interfere with metabolism in this way are likely intermediates in the metabolic pathway of the prime substrate. Therefore the results indicate that benzoate, cyclohexane carboxylate and pimelic acid are intermediates of 3-chlorobenzoate metabolism in WS17. This was later confirmed with another set of experiments where repeated additions of cyclohexane carboxylate and pimelic acid to active 3-chlorobenzoate degrading cultures at different times inhibited 3-chlorobenzoate degradation for short periods. Further support for the contention that the aforementioned compounds are intermediates of 3-chlorobenzoate metabolism in WS17, was found when yeast extract was added to the cultures actively metabolizing 3-chlorobenzoate. Yeast extract (YE) being a good source of carbon increased the culture density but did not inhibit the metabolism of 3-chlorobenzoate.

CELL FREE EXTRACT STUDIES

The best supporting evidence for any biochemical reaction is the presence of a particular enzyme activity in that system. Cell free extract studies for WS17 were taken up with the objective of showing the presence of an enzyme system responsible for the metabolism of benzoate and 3-chlorobenzoate. For benzoate and *p*-hydroxybenzoate metabolism in *R. palustris*, intermediates were confirmed to be in their thioester forms (Geissler et al. 1988; Merkel et al. 1989). Benzoyl-CoA was shown to be the earliest intermediate of benzoate metabolism and was produced by the action of benzoyl-CoA ligase or synthetase (Harwood and Gibson, 1986). The results from whole cell studies presented in the preceding section for benzoate and 3-chlorobenzoate utilization by WS17 showed an indirect evidence for the presence of a benzoate thioester in several samples analyzed through HPLC (Table 5.1 and 5.3). Similarly for other anaerobic bacteria, benzoyl-CoA has been reported as a common intermediate in degradation of a variety of aromatic compounds (Koch and Fuchs 1992).

Benzoyl-CoA was detected in all samples between 0 and 48 hours by HPLC analyses, following incubation of benzoate with cell free extracts from benzoate induced cells in TEA-PO₄ buffer (Table 5.4). Identification of the CoA thioester was by comparing retention time on HPLC to an authentic standard of Benzoyl-CoA. Hydrolysis of these samples resulted in the conversion of benzoyl CoA to benzoate. This benzoate peak was

present in all samples except those for 18 hour and 24 hour, in which benzoyl CoA was still present. This result may be explained by insufficient treatment with NaOH so that the benzoyl CoA from these samples was not converted to benzoate. An unidentified peak at a retention time of 5.1 minutes was also observed in most of these samples.

The presence of benzoyl-CoA was also determined in all samples before thioester hydrolysis, for benzoate induced cells growing on 3-chlorobenzoate in TEA-PO₄ buffer. After the treatment of these samples with NaOH, benzoyl CoA was expected to produce benzoate. However, benzoate was not identified. Instead, the original HPLC peak remained at a retention time identical to that for authentic benzoyl-CoA (Table 5.4). 3-Chlorobenzoate was not found in these samples, whereas an unidentified peak at a retention time of 4.1 minutes was observed in all cases.

As observed from Table 5.4, during the incubation of benzoate with cell free extracts from 3-chlorobenzoate induced cells in TEA-PO₄ buffer, benzoyl-CoA was detected. Whereas, for 3-chlorobenzoate cells growing on 3-chlorobenzoate in TEA-PO₄ buffer, the presence of benzoate was determined in all samples.

These results from cell free extract studies indicated the presence of benzoyl CoA synthetase activity for benzoate induced cells while growing on either benzoate or 3-chlorobenzoate. However, the activity for this enzyme could not be

Sampling Time (hr)	Benzoate induced extract		3-CBa induced extract	
	Ba Deg	3CBa Deg	Ba Deg	3CBa Deg
0	1.040	1.13	(1.72)	2.2*
1	1.7	1.001	1.74	2.489*
2	1.358	(1.11)	1.702	2.64*
5	1.809	1.15	(1.68)	2.379*
18	1.899	1.297	N.D.	2.359*
24	1.152	1.075	2.139*	2.039*
29	1.7 & 2.3*	1.619	2.179*	2.039*
44	1.7 & 1.96	1.5	2.136*	2.52*
48	1.7	1.59	2.089*	2.519*

Note : All samples (aqueous phase) were analysed after TCA precipitation and diethyl ether extraction.

Benzoyl-CoA standard without acid treatment showed retention times in the range of 0.999 to 1.2 min for several samples analyzed. The same standard after acid treatment showed splitting of the peak resulting in several, appearing at retention times between 0.777 and 4.7 min for any single sample.

Retention for Benzoate standard was in the range of 1.99 to 2.43 min.

Deg, Degradation; Ba, Benzoate; 3Cba, 3-chlorobenzoate; * indicate Ba peaks and all others are benzoyl-CoA peaks; (), A tail peak whose retention time was calculated manually from chromatogram.

TABLE 5.4 : CELL FREE EXTRACT STUDIES : RETENTION TIMES OF BENZOATE AND BENZOYL-CoA PEAKS IN Ba AND 3Cba DEGRADATION

measured as the product, benzoyl CoA, was found to be unstable on treatment with TCA. An authentic standard of benzoyl CoA in TEA-PO₄ buffer gave a retention time of 0.999 to 1.2 minutes (a variation typical of that seen for different machine operating periods). On TCA treatment this peak undergoes splitting, forming several peaks appearing between 0.777 and 4.709 minutes. For the same reason the amount of benzoate formed from benzoyl CoA on NaOH treatment could not be measured stiochiometrically. Although quantitative estimation was not possible with this experiment, the qualitaive results obtained here support the earlier observations that the mechanism of 3-chlorobenzoate metabolism involves dehalogenation and formation of a CoA thioester prior to its entry into the benzoate pathway. The presence of benzoyl-CoA as an intermediate is similarly reported for anaerobic degradation of several other compounds (Koch and Fuchs 1992; Lochmeyer et al. 1992). Further metabolism of 3-chlorobenzoate proceeds through benzoyl-CoA, cyclohexane carboxylate (CoA ester) and pimelic acid (CoA ester). Based on these results metabolic pathway for 3-chlorobenzoate metabolism in *R. palustris* WS17 can be proposed as shown in Figure 5.3.

Although no evidence was observed for formation of 3-chlorobenzoyl-CoA conjugate in 3-chlorobenzoate degrading cells, the possibility can not be eliminated based on negative results. However, the ligase enzyme required for formation of this conjugate may be different than that involved in

formation of benzoyl-CoA as the percentage relative activity of this enzyme with 3-chlorobenzoate is reported to be only 1% as compared to that with benzoate (Geissler et al. 1988).

The mechanism of dehalogenation may be non-specific. The first steps in benzoyl CoA metabolism in *R. palustris* involve reduction of the π -bonded aromatic ring to cyclohexane carboxylate-CoA through a series of reductive reactions. The reduction mechanism is unknown, but it is conceivable that reduction involves the addition of hydrogen (in the form of electrons from an electron transport chain, and protons) to the ring. The result of addition of hydrogen to 3-chlorobenzoate may be the fortuitous release of chloride. Such a mechanism, linked to energy conservation as a proton gradient and ultimately ATP, has been proposed for the reductive dehalogenation of 3-chlorobenzoate by the sulfate reducing bacterium *D. tiedjei* DCB-1 (Dolfing 1990).

FIGURE 5.3 : PROPOSED PATHWAY OF 3-CHLOROBENZOATE METABOLISM IN *R. palustris* WS17

KINETICS OF 3-CHLOROBENZOATE DEGRADATION BY *R. palustris*:

Kinetic studies for 3-chlorobenzoate degradation by *R. palustris* WS17 were attempted using batch and continuous culture systems as described in chapter 2. For batch culture kinetic studies WS17 was grown in medium P containing different initial benzoate and 3-chlorobenzoate concentrations (Table 5.5). The substrate utilization rate achieved by each culture at various initial substrate concentrations was used in calculating the values of $\mu_{\max}$ (maximum uptake rate of a compound) and K_s (substrate concentration at which uptake is one half its maximum value) employing Lineweaver Burk Plot (L. B. plot) method. The L. B. plot, $1/\mu$ verses $1/s$ is a linearized form for Michaelis-Menten equation. The slope of this plot gives the value of $K_s/\mu_{\max}$ and the Y-intercept gives value of $1/\mu_{\max}$. This commonly employed method was selected for determination of kinetic constants as opposed to the preferred option of fitting data directly to the Michaelis-Menten non-linear model due to its simplicity. Robinson (1985) presents a good discussion of the application of non-linear regression to kinetic analysis. From the Lineweaver-Burk plots presented in Appendix IV the values obtained for the K_s and $\mu_{\max}$ for benzoate degradation in WS17 were 0.077mM and 1.658 mM/Day respectively. Whereas, for 3-chlorobenzoate degradation the corresponding values were 0.1117 mM and 0.279 mM/Day. Higher $\mu_{\max}$ and lower K_s values for benzoate as compared to those for 3-chlorobenzoate indicated that WS17 has

Benzoate (mM) S_1	$1/S_1$	μ_1 mM/Day	$1/\mu_1$	3CBa (mM) S_2	$1/S_2$	μ_2 mM/Day	$1/\mu_2$
0.25	4	1.263	0.79	0	--	--	--
0.5	2	1.407	0.71	0.25	4	0.193	5.18
1.0	1	1.535	0.65	0.5	2	0.23	4.35
2.0	--	--	--	1.0	1	0.251	3.98

S_1 , benzoate and S_2 , 3-chlorobenzoate concentration; μ_1 , benzoate and μ_2 , 3-chlorobenzoate utilization rate; 3CBa, 3-chlorobenzoate; The values of μ_{max} and K_s determined by Lineweaver Burk Plot Method for benzoate are **1.658 mM/Day** and **0.077mM** respectively. Corresponding values for 3-chlorobenzoate are **0.279 mM/Day** and **0.1117 mM**.

**TABLE 5.5 : DETERMINATION OF KINETIC COEFFICIENTS FOR
BENZOATE AND 3-CHLOROBENZOATE METABOLISM
IN *R. palustris* WS17**

more affinity towards benzoate and utilizes the compound as a preferential growth substrate than 3-chloro-benzoate. These results support the observation that 3-chloro-benzoate alone is not able to support growth in WS17. It is important to note that the values of kinetic constants obtained from batch cultures using sampling intervals of greater than 10% of the generation time do not conform with the assumptions of the Michaelis Menton theory (ie. that the cell numbers remain constant throughout the period of rate measurement). The period of measurement applied here was 24 hours (approximately one generation time) which is typical of slowly growing cultures. Therefore, the constants determined here should be considered approximations of the maximum rate and K_s for benzoate and chlorobenzoate uptake by *R. palustris* WS17.

The main significance of determination of kinetic coefficients is to understand the biochemical activities of the organism in a growth medium. Such results obtained from lab scale studies provide a rational criteria based on biochemical knowledge to design a waste water treatment systems. The kinetic coefficients determined using continuous culture systems are more applicable to real life situations as they simulate with actual waste treatment plants in having a continuous inflow of waste/medium. Several attempts made during this research to study continuous culture system using WS17 in pure culture were not successful. The culture was contaminated even before a steady state was achieved.

CHAPTER 6
ENVIRONMENTAL SIGNIFICANCE OF RHODOPSEUDOMONAS SPECIES
AND THEIR AROMATIC DEGRADATION ABILITIES

INTRODUCTION

Phototrophic bacteria are known to exist in a wide variety of environmental conditions where they are able to obtain their energy for maintenance and growth using CO₂ or organic compounds as carbon sources and employing several types of energy conversion mechanisms including photoautotrophy, photoheterotrophy, chemoheterotrophy and anaerobic fermentation. The members of the family *Rhodospirillaceae*, and in particular *Rhodopseudomonas* species, can employ any of these mechanisms to conduct their metabolic activities (Ormerod and Sirevag 1983, Madigan and Gest 1979, Madigan et al. 1980, Schultz and Weaver 1981). However, photoheterotrophy is their typical mode of substrate utilization. Substrate ranges for these organisms include several aromatic compounds (Harwood and Gibson 1988). Such metabolic versatility and flexibility of metabolic mechanisms allows them to exist under a variety of natural conditions (Biebl and Pfennig 1978). They mainly occur in aquatic environments (Pfennig 1967) and in soils (Sleat and Robinson 1984). In this research I determined the presence of phototrophic Proteobacteria in aquatic environments with samples from a lake, a river and a sewage treatment plant

using "ecocores" to isolate these organisms. Ecocores are model, semi-solid enrichment systems designed to mimic several diffusion gradients typical of anaerobic/aerobic interfaces in nature.

Further, to identify the significance of anaerobic photometabolism in environmental clean-up activities the distribution of the 3-chlorobenzoate catabolic phenotype, and the survival of *R. palustris* WS17 in a mixed bacterial population could be essential investigations. Hence these aspects are studied as presented in the following sections.

MATERIALS AND METHODS

ECOLOGICAL DISTRIBUTION OF RHODOPSEUDOMONAS SPECIES

To investigate the presence of phototrophic bacteria with chloroaromatic degradative abilities in aquatic environments, the following samples were collected during fall season.

- (i) Surface water from Rideau river on campus of Carleton University, Ottawa.
- (ii) Sediments from the same river.
- (iii) Surface sample from an anaerobic sewage treatment plant/anaerobic lagoon (City of Ottawa).
- (iv) Sample from bottom of the same lagoon.
- (v) Water sample collected at the depth of 10 meters from Jacks lake which is situated 60 Km northeast of Peterborough, Ontario.

The samples were used to obtain photosynthetic bacterial populations using an ecocore tube technique, which is an enrichment technique similar in principle to the Winogradsky column. To prepare ecocore tubes semisolid medium P (agar concentration reduced to 0.8%) was seeded with 0.05% (v/v) of the aforementioned samples in 5 separate tubes. After the agar was almost set 10 ml of the respective sample was added on top and the tubes were roled so as to allow the liquid to mix with the solid interface. The tubes were capped and incubated in the presence of red/infrared light as described in Chapter 2. Red coloured colonies developed in the semisolid agar and on

the tube sides after 16 days of incubation. The agar column was sampled under sterile conditions by slicing it and picking up the red colonies. Organisms were separated from agar by a brief sonication in sterile saline. The saline suspension was subjected to dilution plating on yeast extract agar (ATCC medium 112) plates. Colonies from these plates were subcultured several times on plates of minimal medium P (medium P without yeast extract) agar supplemented with 0.15 mM each of biotin and *p*-aminobenzoic acid and either benzoate or 3-chlorobenzoate, or both benzoate and 3-chlorobenzoate as carbon sources to obtain pure culture isolates and to determine the ability of the isolates to utilize these compounds. To investigate the dehalogenation property of the isolate a bromothymol blue pH indicator dye was added to the agar medium. The isolates were also studied as axenic cultures for their ability to degrade these compounds in medium P.

GROWTH OF *R. palustris* WS17 IN MIXED BACTERIAL CULTURES

Growth of *R. palustris* WS17 under environmental conditions was studied using a simulation model. The model was designed for a microbial ecosystem in sediments of a contaminated and stagnant water body where a moderately hydrophobic pollutant such as chlorobenzoate would be expected to be distributed in a concentration gradient throughout the depth of water column, with the maximum concentration in sediments and minimum at the top. Graduated cylinders 27 cm

tall of semisolid agar with benzoate and 3-chlorobenzoate as carbon sources were prepared by layering the aromatic substrates in agar at the bottom of the columns and allowing these substrates to diffuse upward through semisolid agar. A concentration gradient ranging from 0.275 mM to 0.055 mM for benzoate and from 0.339 mM to nil for 3-chlorobenzoate from bottom to top of the columns was achieved. Of the four columns one was inoculated with a WS17 culture (32×10^8 bacteria per mL) (Column B). Column D received a mixed bacterial culture from a sewage treatment lagoon (phototrophs = 32×10^5 bacteria per mL). The third column was inoculated with a 1:1 mixture of the two cultures (WS17 and the sewage sample) (Column C), and the fourth was left uninoculated as a control (Column A). Inoculum in each case was 0.05% of the agar volume. On the top of each agar column was a 5 cm layer of liquid medium P seeded with respective cultures and containing no carbon source. The bottom part of the column (5 cm) was covered with aluminium foil to simulate the dark conditions present in a sediment. The columns were incubated at a distance of 1.75 meter from the light source. At the end of incubation period samples were collected from agar columns using a sterile hollow glass rod as a corer. Agar contents from the glass rod were divided in 14 parts along the length (12 parts of 2 cm each from top i.e. sample numbers 3 to 14; and 2 parts of 1.5 cm each i.e. sample numbers 15 and 16). Agar from each part was transferred to an already weighed eppendorf tube and the tube was weighed again

(average weight of each part was 5.5 to 5.6 grams for 2 cm long sample and 4.2 to 4.4 grams for 1.5 cm long sample). The water content of the semisolid agar sample was separated by centrifugation at 13000 rpm for 10 min. The aqueous phase was analyzed by HPLC to determine benzoate and 3-chlorobenzoate concentration through the depth of the column. Microorganisms from the agar sample were eluted in sterile distilled water by a brief sonication. This bacterial suspension was plated on yeast extract agar medium (ATCC medium 112) to obtain bacterial counts at various depths in the column.

HALOGENATED AROMATIC SUBSTRATE RANGE DETERMINATION

The ability of WS17 to metabolize or to cometabolize various halogenated aromatic compounds was investigated using medium P containing the respective compound alone as a carbon source or with benzoate as a cosubstrate. The culture was acclimatized for the process over a period of at least 7 to 8 weeks. Growth of the culture and disappearance of substrate were monitored as described in Chapter 2. To obtain better resolution on the chromatograms for certain compounds like 2-chlorobenzoate and 2,4-Dichlorobenzoate, the solvent composition was changed from the original 1:1 ratio to 4:1 ratio of 1% acetic acid to methanol.

RESULTS AND DISCUSSION

ECOLOGICAL DISTRIBUTION OF *RHODOPSEUDOMONAS* SPECIES

Metabolic versatility and flexibility of energy conversion mechanisms in the non-sulfur Proteobacterial group of phototrophs are responsible for their wide spread ecological distribution, especially in the environments where anaerobic and euphotic zones coincide. Thus the preferred habitats for these organisms are small eutrophic lakes, flooded soils and anaerobic surface sediments in ponds and ditches.

During these studies the presence of *Rhodopseudomonas* species in various ecosystems was investigated by subjecting the samples collected from different aquatic systems to enrichment by the ecocore tube technique. The technique is similar in principle to the Winogradsky Column and is based on the establishment of different microenvironment layers from the bottom to the top of the tube. Each of the ecocore tubes at the end of the incubation period showed the presence of green algal (oxygenic) phototrophs in the liquid column on top as well as at the interface of solid and liquid phases (Figure 6.1). Underneath was a region of red layers and/or microcolonies indicating the presence of anaerobic phototrophs. Figure 6.1 shows a high density of algal biomass for the river sediment sample as compared to the others, for the reason that the river sediment sample was used without dilution and the other samples were diluted 10^2 times. For the

**FIGURE 6.1 : GROWTH OF PHOTOTROPHIC BACTERIA
IN ECOCORE TUBES**

Tubes were filled with semi-solid medium P (with benzoate and 3-chlorobenzoate) made by including the inoculum before the agar set. The semi-solid agar was then topped up with inoculum, leaving an air space of about 1 inch. The caps were loosely attached to allow the diffusion of air into the tubes, and they were placed in the light. From left to right the inocula were:
Jack's Lake (sample 1); Jack's Lake (sample 2); Rideau River sediment; and Rideau River surface waters.

FIGURE 6.1 : GROWTH OF PHOTOTROPHIC BACTERIA
IN ECOCORE TUBES

sewage treatment lagoon sample (not shown in Figure 6.1) beneath the phototrophic region a few blackish brown patches, most probably due to the growth of sulfate reducers, were observed. These observations show identity with the positioning of phototrophs in natural ecosystems where they play a significant role in the interconnection of carbon and sulfur cycles. Phototrophs can fix carbon dioxide through photoautotrophy and can utilize organic compounds through photoheterotrophic metabolism, while sulfide and thiosulfate are used by sulfur group of phototrophs. The extrapolation of such lab scale studies to natural ecosystems should be, however, made very carefully as the natural environments are far more complex than these simplified versions. The ecocore distribution of temperature, pH, substrate concentrations and the uninterrupted availability of constant illumination will not reproduce the complex ranges of these parameters in nature. The enrichment approach serves only to indicate the presence of particular physiological groups of bacteria in a sample.

The results from these studies showed the presence of anaerobic photosynthetic bacteria in all samples tested, even in surface water samples from the river, which is an aerobic environment. Several enrichments of phototrophs (red colonies on Yeast Extract medium agar plates) obtained from these samples were indistinguishable from each other except for their colony size. All the enrichments were mixed cultures of

motile cocci, 0.5-0.7 μm in size; nonmotile rods nearly 2 to 3 μm long and highly motile rods resembling *R. palustris*, the dominant cell types of the mixed culture. These results support the earlier observation regarding all aquatic environments being habitats of phototrophs (Pfennig, 1967).

Table 6.1 shows the number and percentage of mixed culture enrichments from different sources growing on various media. Only a few phototrophic enrichment cultures were recovered from the sewage treatment plant, especially from bottom samples. The probable reason may be that the waste water from the treatment lagoon was almost a black mass and the top was like a black crust. This must prevent the penetration of sufficient light through the depth of the lagoon, resulting in either the absence of phototrophs, or a low number of these.

Dehalogenation activity was observed in several of these enrichment cultures by noting the pH change of the medium as HCl was released. The bromothymol blue indicator changes from blue to greenish blue or yellow depending on the extent of dehalogenation.

These mixed culture enrichments, when grown on benzoate and 3-chlorobenzoate medium under anaerobic conditions, resulted in the isolation of a few axenic cultures. All of these pure culture isolates were strains of *R. palustris* and were able to degrade these compounds in liquid medium P as indicated by the results presented later in Table 6.5.

Samples	Total isolates	On Y.E. Med		On Ba Med		On 3CBa Med	
		#	%	#	%	#	%
Jacks Lake	216	216	100	214	99	214	99
River (Surface)	168	150	89	98	58	84	50
River (Sediment)	240	177	74	163	68	40	17
Lagoon (Top)	120	108	90	61	51	10	8
Lagoon (Bottom)	48	46	96	32	67	25	52

Y. E. Med- Yeast extract medium ATCC 112; Ba Med- Minimal medium P containing only benzoate; 3CBa Med- Minimal medium P containing 3-chlorobenzoate.

**TABLE 6.1 : MIXED CULTURE PHOTOTROPHIC ENRICHMENTS FROM
AQUATIC ENVIRONMENTS GROWING ON
DIFFERENT MEDIA**

The results of these experiments show that *Rhodopseudomonas* species are the dominant anaerobic phototroph enriched under several environmental conditions and that they are able to dehalogenate and degrade 3-chlorobenzoate in the laboratory. The results imply that the 3-chlorobenzoate catabolic phenotype is a common feature of *R. palustris* metabolism, despite the observation that the ATCC Type strain (ATCC 17001) was not able to degrade 3-chlorobenzoate without acclimatization.

RANGE OF SUBSTRATES

Anaerobic niches in the environment, besides being habitats for phototrophs, serve as sinks for several aromatic and non-aromatic pollutants released due to various industrial and human activities. These aromatic pollutants, in particular the halogenated ones, are of great concern because of their toxicity and widespread occurrence. The degradation of simple aromatics by phototrophs, especially by the members of the family *Rhodospirillaceae*, is reported earlier (Harwood and Gibson 1988). However, chloroaromatic degradation by phototrophs was not known until the isolate *R. palustris* WS17 was found to degrade 3-chlorobenzoate. This newly isolated strain was further studied to determine its ability to degrade several other haloaromatic, hydroxy, methoxy and amino substituted compounds in the presence and absence of the cosubstrate benzoate. A WS17 culture actively growing in

medium P was used as an inoculum. Acclimatization of the WS17 culture was attempted for a period of 7 to 8 weeks through 2 transfers on medium P containing the compound to be tested. Table 6.2 shows the percentage of initial concentration of substrate being degraded by WS17 at each stage of acclimatization.

The results presented in Table 6.2 show that acclimatization did not help to enhance the degradative capacity of WS17 for mono- or di-chlorobenzoates, either in the presence or absence of benzoate. For both cultures, degradation was progressively reduced with substrates like 4-chlorobenzoate (4 CBa), 2,4-dichlorobenzoate (2,4 DiCBa) and 3,4-dichlorobenzoate (3,4 DiCBa), whereas, the degradative capacity was almost constant for 2-chlorobenzoate (2 CBa), 2,5-dichlorobenzoate (2,5 DiCBa) and 3,5-dichlorobenzoate (3,5 DiCBa) on transfer to a fresh medium during the process of acclimatization. Comparison of results for cultures with and without benzoate in the medium showed that only for 2-chlorobenzoate did the percentage degradation increased from a range of 51-56% (culture A to culture C) to a range of 90-95% by addition of benzoate to the medium.

The decrease in percentage degradation can probably be explained as follows. WS17 used as an inoculum possessed an active enzyme system for benzoate and 3-chlorobenzoate degradation and in the initial culture (Culture A) showed the maximum percentage degradation of the other chlorobenzotes

Compounds	% Degradation in Absence of Benzoate			% Degradation in Presence of Benzoate		
	Cult A	B	C	Cult A	B	C
2 CBa	51	56	53	95	95	90
4 CBa	42	36	11	42	28	16
2,4 DiCBa	43	17	15	45	22	28
2,5 DiCBa	34	43	33	33	33	32
3,4 DiCBa	43	16	18	29	16	18
3,5 DiCBa	16	13	18	24	13	22
3 BrBa	43	46	65	69	63	91
4 OHBa	100	100	100	100	100	100
Vanillic Acid	56	52	83	83	85	98
Isovanillic Acid	29	43	57	51	49	59
Aniline ^a	34	37	56	48	52	59
3 Chloro- Aniline ^a	20	23	38	47* (31)	32* (54)	59* (51)

*, Aniline was used as a cosubstrate for 3-chloroaniline metabolism.; (), corresponding aniline degradation.; @ Initial concentration of aniline 0.4 mM and that of 3-chloroaniline 0.2 mM.; Initial concentration of all substrates was 0.5 mM except aniline and 3-chloroaniline; Vanillic acid, 4-hydroxy,3-methoxy benzoate.; Isovanillic acid, 3-hydroxy,4-methoxy benzoate.; Cult A is initial culture without acclimatization.; Cult B is culture after first transfer.; Cult C is culture after second transfer.

TABLE 6.2 : DEGRADATION OF SUBSTITUTED AROMATICS BY

R. palustris WS17

tested. However, on each transfer to a fresh medium, the number of active chlorobenzoate degrading cells must have been reduced due to growth and dilution. The new compound tested (for example 4-chlorobenzoate) was most probably unable to support growth or contribute reducing equivalents during phototrophic metabolism, which resulted in a further decrease in the number of acclimated cells, eventually leading to decreased degradation with each transfer. In the presence of benzoate, though the reducing potential was available for phototrophic growth of WS17, the cosubstrate benzoate probably could not maintain an active population of 4-chlorobenzoate degraders. This may be due to specificity of the enzymes of the 3-chlorobenzoate pathway induced in the presence of benzoate for *ortho*- and *meta*-substituted haloaromatics. These results also suggest that the compounds like 4-chlorobenzoate, 2,4-dichlorobenzoate and 3,4-dichlorobenzoate undergo only limited dehalogenation and do not act as growth substrates for WS17.

The sulphate reducing bacterium *D. tiedjei* strain DCB-1 gains energy from the dehalogenation of 3-chlorobenzoate without utilizing the product of dehalogenation (Dolfing 1990). Whether *R. palustris* WS17 gains any metabolically useful energy from the anaerobic dehalogenation of these chlorinated substrates is unknown.

For compounds 2-chlorobenzoate, 2,5-dichlorobenzoate and 3,5-dichlorobenzoate the percentage degradation was neither

reduced nor enhanced due to acclimatization, probably because these compounds were able to support the growth of WS17 to some extent thus maintaining a constant population of active dehalogenating organisms.

Results from Table 6.2 for chlorobenzoates show that only 2-chlorobenzoate was significantly metabolized by WS17 in the presence of benzoate. However, further substitution with a second chlorine atom in either position 4 or 5 halted or reduced degradation of the compound. A similar trend was observed for 3-chlorobenzoate. The reduction in percentage degradation when a second chlorine is placed in position 5 is more than that for position 4. For the first chlorine in position 3 and second chlorine in position 5 (3,5-dichlorobenzoate) the percentage degradation is low.

Degradation studies for 3-bromobenzoate show a positive effect of acclimatization as well as for the presence of benzoate in the medium, although the degradation is low as compared to 3-chlorobenzoate. The possible explanation for this may be that the WS17 culture was acclimatized for 3-chlorobenzoate degradation and not for 3-bromobenzoate degradation. However, cross adaptation with the substrate analogue may be responsible for 3-bromobenzoate degradation observed in WS17. A similar phenomenon of cross adaptation was reported by Urbanek et al. (1989).

p-Hydroxybenzoate was degraded most efficiently by WS17 and did not require any acclimatization period. The presence

of the cosubstrate benzoate in the medium however reduced the efficiency of WS17 to metabolize 4-hydroxybenzoate. This can be explained by the fact that both the substrates are efficiently taken up by WS17 but benzoate may be a preferable substrate. It is quite likely that benzoate and 4-hydroxybenzoate compete for the same enzyme system or benzoate is an intermediate of the 4-hydroxybenzoate metabolic pathway and hence shows intermediate inhibition leading to reduction in the rate of 4-hydroxybenzoate degradation (from 0.33 mM per day to 0.20 mM per day).

An efficient degradation of *p*-hydroxybenzoate by WS17 suggests an electrophilic attack on the aromatic ring during its anaerobic degradation. Substituents such as amino, methyl and hydroxyl are electron contributing groups, hence increasing the electron density on the aromatic nucleus which makes the ring more susceptible to electrophilic attack.

The results from Table 6.2 show obvious reductions in degradation with the presence of methyl and hydroxyl groups together as compared to the compound with hydroxyl group alone. For vanillic acid (4-hydroxy-3-methoxy benzoic acid) and isovanillic acid (3-hydroxy-4-methoxy benzoic acid), the presence of cosubstrate and acclimatization enhanced the degradation. The exchange of positions for the hydroxyl and methyl groups, however, reduced the degradation capacity by nearly 40% in isovanillic acid. This indicates the maximum efficiency of electrophilic attack occurs when a hydroxyl

group is in the *para* position to the carboxy group in benzoate. To understand the effect of a change in position of a hydroxyl group from *para* to *meta*, and the effect of an additional methyl substituent, on anaerobic degradation of the aromatic ring, further studies are necessary.

In the case of amino substituted aromatics (aniline and 3-chloroaniline) acclimatization and the presence of a cosubstrate in the culture medium enhanced the degradation. For 3-chloroaniline degradation, aniline was used as a cosubstrate instead of benzoate. Similar to the other cases, the presence of a chlorine substituent reduced the degradation of aniline by WS17. The presence of chlorine, a highly electronegative substituent on the aromatic ring, is expected to reduce the susceptibility of the ring to an electrophilic attack. Most probably for this reason the degradation of 3-chloroaniline was lower than for aniline. This observation parallels the observation of slower degradation of 3-chlorobenzoate relative to benzoate, and supports an initial electrophilic attack on the aromatic ring in the anaerobic degradation of hydroxy, methoxy and amino substituted aromatics.

For dehalogenation, an electrophilic attack on the aromatic ring has not been shown. Dolfing and Tiedje (1991) suggested a nucleophilic attack on the aromatic ring as one of the reaction steps of the dehalogenation reaction in *D. tiedjei*.

The substrate range for WS17 as determined by these experiments includes monohalogenated compounds like 2-chlorobenzoate, 3-chlorobenzoate, 3-bromobenzoate; hydroxy and methoxy compounds like *p*-hydroxybenzoic acid, vanillic acid, isovanillic acid; and amino substituted compounds like aniline and 3-chloroaniline. Dihalogenated compounds were not significantly degraded by WS17 either in the presence or in the absence of benzoate, however they undergo dehalogenation to a limited extent. As 2-chlorobenzoate and 3-chlorobenzoate are cometabolized, whereas 4-chlorobenzoate could not be metabolized by WS17, the indication is that chlorines in position 2 or 3 are removed easily as compared to position 4. In the case of dihalogenated compounds like 2,4-dichlorobenzoate, after the removal of chloride from position 2, the product formed was 4-chlorobenzoate which could not undergo further metabolism in WS17. Such stepwise dehalogenation is reported for polychlorinated aromatics in methanogenic consortia by Suflita et al. (1982). As the product of dehalogenation was not degraded further by WS17, generation of hydrogen, a source of reducing equivalents necessary for further dehalogenation, could not occur. A low production of hydrogen and a delayed growth response was observed by Dolfig and Tiedje (1991) during degradation of 3,5-dichlorobenzoate in *D. tiedjei*.

In the presence of phenol, 3-chlorophenol or 4-chlorophenol even at very low concentrations (0.001 mM) WS17

did not survive.

The effect of substituents on aromatic degradation under aerobic conditions is well documented (Schmidt and Knackmuss 1980; Dorn and Knackmuss 1978a and b; Reineke and Knackmuss 1978). However, little is known about their effect on anaerobic dehalogenation. In general, substituents affect anaerobic degradation of aromatics through several means by:

- (i) influencing both the chemistry and biology of the process,
- (ii) affecting the generation of reducing power,
- (iii) inhibiting the formation of an enzyme substrate complex,
- (iv) affecting the energy of activation.

GROWTH OF *R. palustris* WS17 IN MIXED BACTERIAL CULTURE

As suggested by the results from the previous section, *R. palustris* WS17 is an organism with the ability to metabolize a wide range of aromatic compounds including halogenated aromatics. Due to this distinguishing characteristic it is important to understand how well WS17 can compete and survive in mixed bacterial cultures. This is essential to exploit its haloaromatic degradation property for environmental pollution control procedures.

A composite sample from an anaerobic sewage treatment lagoon (city of Ottawa) was used as a representative mixed bacterial population existing under anaerobic conditions. The survival of WS17 when mixed with this culture and allowed to grow was determined by observing the pattern of growth, and

the numbers of photosynthetic anaerobes in the layers of the cores compared to the numbers observed when the sewage inoculum was incubated alone. By observing substrate degradation in a gradient of benzoate and 3-chlorobenzoate concentrations, the contribution of WS17 could also be assessed. In each inoculated column a red band of photosynthetic bacterial growth was seen at a level of 5 to 6 cm from the bottom and diffused growth was present throughout the depth of column as shown in Figure 6.2. Growth in column D (lagoon culture) was, however, observed in patches. This correlates well with the ecocore enrichment results reported above. Intensity of the red band, as well as growth in the overall column was highest for column C (WS17 plus lagoon inoculum) and lowest for column B (WS17 pure culture inoculum). Bacterial counts obtained with the dilution plate technique supports these observations although it is not a reliable method for cluster forming bacteria like *R. palustris*. WS17, as it forms rosette clusters, will yield a lower count than the existing number. Table 6.3 provides information regarding bacterial counts for phototrophs (number of red colonies observed on yeast extract agar medium plates following incubation under anaerobic conditions in presence of light) in samples collected at various depths in the respective columns. For WS17, sample numbers 1 and 2 from the liquid phase on top of the columns showed higher counts than those from the semi-solid agar phase below. This may be

**FIGURE 6.2 : GROWTH OF *R. palustris* WS17 IN
A MIXED BACTERIAL CULTURE**

R. palustris WS17 was inoculated into columns of semi-solid agar which contained a diffusion gradient of benzoate and 3-chlorobenzoate (highest concentration at the bottom of the tubes).

Tube 1 (left) was uninoculated;

Tube 2 contained WS-17 alone;

Tube 3 contained WS-17 and Sewage

Treatment Plant mixed bacteria;

Tube 4 contained Sewage Treatment Plant
bacteria alone.

Sample # (position)	CFU, Column B # x 10 ⁶	CFU, Column C # x 10 ⁶	CFU, Column D # x 10 ⁶
1 (32)	400	620	160
2 (28)	460	580	220
3 (27)	15	219	245
4 (25)	11	139	181
5 (23)	138	152	253
6 (21)	58	240	256
7 (19)	34	422	237
8 (17)	30	304	232
9 (15)	19	314	239
10 (13)	10	907	196
11 (11)	58	483	358
12 (9)	137	455	334
13 (7)	61	71	206
14 (5)	193	96	58
15 (3)	15	60	46
16 (1.5)	77	162	88

(), position of sample in column from the bottom in cm. e.g. 13 cm indicates the position as 11 to 13 cm.; CFU, colony forming units.; Column B, inoculated with *R. palustris* WS17.; Column C, inoculated with lagoon sample and WS17.; Column D, inoculated with lagoon sample.

**TABLE 6.3 : COLONY FORMING UNITS OF PHOTOTROPHS PRESENT ALONG
THE DEPTH IN DIFFERENT COLUMNS.**

because in liquid medium cells are loosely associated and swimmers are highly motile, and hence are better distributed than in solid agar.

At the beginning of the experimental period benzoate (1.0 mM) and 3-chlorobenzoate (0.5 mM) were present only in the semisolid agar medium added at the bottom of the column (3 cm layer). Diffusion of these substrates occurred through the agar column over the period of incubation forming a concentration gradient which was represented by the substrate concentrations at various depths in the uninoculated column (Column A) as given in Table 6.4A and B. The tables also show concentration gradients for benzoate and 3-chlorobenzoate at the end of the incubation period (i.e. remaining substrate concentration) in inoculated columns (Columns B to D). The amount of substrate utilized by cultures in any experimental column was calculated by subtracting the remaining concentration at various depths in the column from that of the blank.

In each column substrate utilization can be explained in two parts.

(i) Up to 11 cm from the bottom of column where most of the degradation was seen (samples from this part are numbered 11 to 16).

(ii) From above 11 cm to the top of agar column (samples from this part are numbered 3 to 10).

Sample # (Position)	Column A	Column B	Column C	Column D
1 (32)	0.055	--	--	0.001
2 (28)	0.054	0.001	--	--
3 (27)	0.055	0.029	0.065	0.057
4 (25)	0.056	0.032	0.062	0.057
5 (23)	0.064	0.029	0.063	0.073
6 (21)	0.067	0.031	0.072	0.075
7 (19)	0.078	0.033	0.070	0.079
8 (17)	0.0999	0.032	0.07	0.092
9 (15)	0.115	0.032	0.07	0.103
10 (13)	0.129	0.029	0.075	0.093
11 (11)	0.169	--	0.071	0.089
12 (9)	0.1799	--	0.073	0.091
13 (7)	0.185	--	0.076	0.095
14 (5)	0.228	--	0.083	0.088
15 (3)	0.243	--	0.079	0.096
16 (1.5)	0.275	--	0.079	0.098

Column A, uninoculated; Column B, inoculated with WS17; Column C, inoculated with WS17 + lagoon culture; Column D, inoculated with lagoon culture.

**TABLE 6.4 A : BENZOATE CONCENTRATIONS (mM) AT VARIOUS DEPTHS
IN COLUMNS AT THE END OF INCUBATION PERIOD**

Sample # (Position)	Column A	Column B	Column C	Column D
1 (32)	--	--	--	--
2 (28)	0.0063	--	0.009	0.005
3 (27)	0.031	0.028	0.029	0.03
4 (25)	0.032	0.038	0.027	0.032
5 (23)	0.035	0.042	0.024	0.032
6 (21)	0.061	0.043	0.06	0.06
7 (19)	0.075	0.071	0.069	0.077
8 (17)	0.081	0.086	0.085	0.099
9 (15)	0.086	0.084	0.077	0.071
10 (13)	0.079	0.082	0.128	0.073
11 (11)	0.186	0.152	0.147	0.093
12 (9)	0.197	0.163	0.174	0.168
13 (7)	0.315	0.219	0.234	0.183
14 (5)	0.328	0.224	0.266	0.231
15 (3)	0.326	0.259	0.261	0.313
16 (1.5)	0.339	0.247	0.282	0.357

Column A, uninoculated; Column B, inoculated with WS17; Column C, inoculated with WS17 + lagoon culture; Column D, inoculated with lagoon culture.

**TABLE 6.4 B : 3-CHLOROBENZOATE CONCENTRATIONS (mM) AT
VARIOUS DEPTHS AT THE END OF
INCUBATION PERIOD**

Significant degradation was observed only in the bottom samples, sample numbers 11 to 16 (up to 11 cm from bottom). In these samples WS17 (Column B) utilized 100% of the benzoate and 20 to 35% of the 3-chlorobenzoate available. For the same samples benzoate utilization in column C (lagoon + WS17) was 57 to 71% and 3-chlorobenzoate utilization was 16 to 20%. Similarly in column D (lagoon culture) the percentages of utilization were 47 to 64% for benzoate and 4 to 14% for 3-chlorobenzoate.

The samples collected from the upper part of the agar columns, sample numbers 3 to 10 (from above 11 cm to the top of agar column) showed only benzoate metabolism by WS17. Whereas, other cultures (from columns C and D) showed no significant metabolism of either benzoate or 3-chlorobenzoate in these samples.

In the liquid layers on top of agar column (sample numbers 1 and 2), benzoate and 3-chlorobenzoate were actively metabolized by WS17. For the other two cultures (from columns C and D) although benzoate was metabolized, 3-chlorobenzoate remained in the medium (in sample number 2). In sample number 1 no 3-chlorobenzoate was present in water column, which may be due to poor solubility of the compound because of which it could not reach to the top of the water layer.

Each of the columns were inoculated with the same amount of inoculum, however, the composition of the inocula were different. Hence variability in benzoate and 3-chlorobenzoate

degradation in any two different columns can be accounted for the variable composition of the inoculum.

The results show that the maximum amount of benzoate and 3-chlorobenzoate were utilized in column B (inoculated with WS17) and the minimum in column D (inoculated with lagoon culture). Higher amounts of substrate utilization in column C (inoculated with lagoon culture + WS17, 1:1), compared to column D, indicate that the degradation was enhanced by the presence of WS17. The results also indicate that WS17 is able to grow within a mixed anaerobic culture and retain its property to degrade 3-chlorobenzoate.

As seen in Figure 6.2, a heavy growth was apparent in column C (lagoon + WS17), however, substrate utilization was less than that observed in column B (WS17 alone). This may be due to the presence of a high number of phototrophs, but a low number of aromatic degraders, in column C. In this column only 50% of the inoculum was WS17, whereas, in column B all the observed growth was due to WS17 and was therefore able to metabolize higher amount of benzoate and 3-chlorobenzoate.

Degradation of 3-chlorobenzoate was also observed to a small extent in column D (lagoon culture), which indicates the presence of 3-chlorobenzoate degrading organisms in the lagoon. The lagoon sample, being a mixed culture of anaerobic organisms other than phototrophs, may carry organisms competent for 3-chlorobenzoate degradation which may be either phototrophs or other anaerobes. The experimental conditions,

however, were similar to that used for phototroph enrichment. Hence 3-chlorobenzoate degradation observed for this column can be expected by phototrophs, and most probably by indigenous *R. palustris*. *R. palustris* is the only anaerobic phototroph known to degrade aromatic compounds (Imhoff and Truper 1989). The results support the presence of phototrophs in a wide variety of aquatic environments.

Extrapolation of these results to natural systems is constrained by the conditions provided for phototroph enrichment. Similarly the light intensity was constant through the depth of the columns. This is clearly not the case under natural environmental conditions. Attempts have always been made to improve lab scale systems for their direct application to natural environmental conditions (Gibson and Suflita 1986).

DISTRIBUTION OF 3-CHLOROBENZOATE METABOLIC PHENOTYPE

Results for investigations of the ecological distribution and survival of *Rhodopseudomonas palustris* indicate the widespread distribution of the organism and the common occurrence of the 3-chlorobenzoate degradation phenotype. Studies on the 3-chlorobenzoate metabolic phenotype in the various phototrophic isolates recovered from different aquatic environments (Table 6.5) show that 3-chlorobenzoate was cometabolized by all strains tested, in the presence of benzoate as a cosubstrate. However, 3-chlorobenzoate degradation without benzoate was not observed.

<i>R. palustris</i> Strain	3Cba metabolized (mM)
ATCC17001	0.0275
WS17	0.5
CGA001	0.09
CGAO33	0.00
CGA047	0.00
RPS-5	0.498
JL78	0.341
JL86	0.480
JL88	0.469
JL94	0.476
JL95	0.465
JL96	0.437
RS51	0.476
RS52	0.466
RS71	0.433
RS72	0.496

3CBa, 3-Chlorobenzoate; Strains are described in chapter-2
Initial concentration of 3-chlorobenzoate was 0.5mM and incubation period was 18 days.

Note : Column 2 indicates concentration of 3-chlorobenzoate degraded in presence of 1mM of benzoate.

TABLE 6.5 : 3-CHLOROBENZOATE DEGRADATION BY STRAINS OF

R. palustris

The results with several isolates from various samples indicated that the presence of *R. palustris* expressing 3-chlorobenzoate degradation activity in aquatic environments was not a rare phenomenon. In waste treatment lagoons operated by a number of different industries the existence of large blooms of purple photosynthetic bacteria can be observed (Federle 1989). Wastewaters held in lagoons which are open to sunlight and which are receiving high loads of organic matter, such as pulp and paper mill effluents, food manufacturing waste streams and detergent wastes, often support copious mats of these bacteria which persist even following efforts to aerate the water. In these environments complex mixtures of aromatic substrates, in some cases including halogenated aromatics, may be important substrates for phototrophic growth. *R. palustris* may contribute to the removal of chlorinated aromatics under these conditions. These observations suggest that the 3-chlorobenzoate degradation property of *R. palustris* is an important attribute and can be exploited in waste water treatment systems.

CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND FUTURE PROSPECTS

Chlorinated aromatics are major environmental pollutants. They are released in natural systems in the form of pesticides, several industrial chemical products and effluents from the industries involved in production of such chemicals. Most of them are toxic and tend to persist in the environment due to their xenobiotic nature. However, biodegradation plays an important role in controlling the pollution caused by these compounds. Dehalogenation is an essential step in reducing the toxicity and increasing their biodegradability. Reductive dehalogenation is an important phenomenon mainly occurring under anaerobic conditions, and is the only known biodegradation mechanism for certain pollutants like polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), and pentachlorophenol (PCP). Hence any research involving studies with anaerobic dehalogenating organisms and the mechanism of dehalogenation or degradation of haloaromatics is a step ahead in understanding the environmental fate of pesticides and industrial chemicals. Also it is a step towards bioremediation of hazardous organic pollutants.

Considering this fact the research presented in this thesis is both original and significant in the field of biodegradation. This research is the first report of the degradation of halogenated aromatic compounds by

photosynthetic bacteria under anaerobic conditions.

Phototrophs in general and the members of family *Rhodospirillaceae* in particular, have a range of metabolic abilities and flexibility of energy conversion mechanisms (Harwood and Gibson 1988; Dutton and Prince 1978) which help them to inhabit a variety of environments. Photoheterotrophy is their main metabolic mechanism and their substrate range includes several simple aromatics.

During this research a mixed, phototrophic dehalogenating culture was enriched under anaerobic conditions and an isolate of a 3-chlorobenzoate degrading phototroph was obtained. The newly isolated phototroph was identified as *Rhodopseudomonas palustris* (strain WS17) based on morphological, biochemical and genetic characterization as described in Chapter 3. *R. palustris* WS17 was further employed during this research to investigate the mechanism of 3-chlorobenzoate metabolism and to study the kinetics of the degradation process. The natural occurrence and environmental significance of the 3-chlorobenzoate metabolic phenotype in phototrophs isolated from various freshwater and wastewater environments were also assessed.

R. palustris WS17 was found to degrade 3-chlorobenzoate in the presence of benzoate under anaerobic conditions with light. The experiments presented in Chapter 4 identify these requirements of 3-chlorobenzoate metabolism in the organism. Radioisotopic studies with [U-¹⁴C]-3-chlorobenzoate showed

incorporation of 60% of radioisotope (ring carbon) in biomass and 20% in CO₂ produced during the process, with 20% remaining as unutilized substrate. These results suggested that the chlorinated substrate is capable of serving as a significant source of carbon for biosynthesis. Further studies conducted to identify the mechanism of 3-chlorobenzoate metabolism indicated that the compound underwent dehalogenation, most probably as the first step in the metabolic pathway. This observation combined with that of radioisotopic studies indicated that the product of dehalogenation was further utilized by WS17 and was thus able to produce reducing potential necessary for growth and further reductive dehalogenation. Thus a single organism was capable of 3-chlorobenzoate degradation.

Degradation of 3-chlorobenzoate is known for a defined methanogenic consortium (Mohn and Tiedje 1992). A dehalogenating organism, *D. tiedjei* DCB-1, dehalogenates the compound and the product is then utilized by other members of the consortium. *D. tiedjei* however, can not utilize the product, benzoate, and depends on other microorganisms in the group to provide reducing potential necessary for growth (in the form of simple organic acids like lactate and pyruvate). The independence of the phototrophs described in this research for the degradation of chlorobenzoates, as opposed to the case of the methanogens which must function in consortia, may provide them an advantage over methanogens.

Although, *R. palustris* WS17 does not depend on other organisms for reductive dechlorination of 3-chlorobenzoate, it requires the presence of a cosubstrate in the medium. Benzoate was determined to be the best choice as it supported the maximum rate of degradation for the compound. The presence of benzoate along with chlorobenzoate is not uncommon in nature, hence the 3-chlorobenzoate degradation phenotype of *R. palustris* WS17 may be of environmental significance.

The role of benzoate in 3-chlorobenzoate metabolism by WS17 was established as an inducer of the pathway. Once the enzyme system was activated by benzoate, 3-chlorobenzoate was efficiently degraded by WS17. Compounds like cyclohexane carboxylate, capable of inducing the entire benzoate reductive pathway, including the first enzyme of the benzoate pathway, benzoyl-CoA ligase, were also able to induce 3-chlorobenzoate metabolism in WS17 (Hutber and Ribbons 1983; Blakley 1978). This observation indicates that most probably benzoate and 3-chlorobenzoate utilize the same reductive enzyme system, including at least the first enzymes of the pathway. The specificity of the enzyme benzoyl-CoA ligase is an important aspect to be studied. If the enzyme is non-specific for benzoate then it will be able to combine with both 3-chlorobenzoate as well as benzoate. Thus forming a 3-chlorobenzoyl-CoA complex prior to dehalogenation. Alternatively, the product of direct dehalogenation, benzoate, may then react with benzoyl-CoA ligase. The results obtained during whole

cell studies using GCMS and HPLC analyses showed the intracellular presence of 3-chlorobenzoate and benzoate thioester. Cell free studies indicated the presence of benzoyl-CoA-ligase activity for benzoate induced cells while growing on either benzoate or 3-chlorobenzoate. This indicated a non-specific nature of the enzyme and suggested that 3-chlorobenzoate was dehalogenated to benzoate, either directly, or possibly as a CoA thioester. Once the compound was dehalogenated it would readily enter the benzoate pathway. This hypothesis was supported by the observations of intermediate inhibition studies which indicated benzoate, cyclohexane carboxylate and pimelic acid as the intermediates in 3-chlorobenzoate pathway. Thus the 3-chlorobenzoate degradation pathway in *R. palustris* WS17 may be depicted as in Figure 5.3.

In addition to 3-chlorobenzoate, WS17 was found to degrade several other halogen, hydroxy, methoxy and amino substituted aromatic compounds like 2-chlorobenzoate, 3-bromobenzoate, *p*-hydroxybenzoate, vanillic acid, isovanillic acid, aniline and 3-chloroaniline. This extended the substrate range of WS17 and further enhanced its importance for bioremediation processes under anaerobic conditions. Although degradation of monohalogenated compounds was observed in WS17, it could not degrade dichlorinated compounds like 2,4-dichlorobenzoate completely. Incomplete reductive dehalogenation, due to a lack of reducing potential, could be the reason for the organism

not being able to utilize the compound as its growth substrate. Further studies under hydrogen pressure or in the presence of acetate as a source of reducing power are warranted to investigate the ability of WS17 to dehalogenate di- and polychlorinated compounds. A recent development, published after the research was completed for this thesis, was the observation of slow dechlorination of tetrachlorobiphenyl by a phototrophic enrichment culture, apparently dominated by purple non-sulfur bacteria (Montgomery and Vogel 1992). Although the amount of tetrachlorobiphenyl dehalogenated was small (about 10 μ M over a 350 day period), this is a promising report supporting the ability of phototrophs to dehalogenate polychlorinated compounds under anaerobic conditions.

This metabolic versatility of the purple non-sulfur group of phototrophs may be responsible for their occurrence in a variety of environments, especially where anaerobic and euphotic zones coincide. Several samples representing different aquatic environments were tested during this research to investigate the ecological distribution of *Rhodopseudomonas* species. The results with a number of isolates from these samples indicated that the presence of *R. palustris* with the ability to degrade 3-chlorobenzoate in environmental samples was not a rare phenomenon. *R. palustris* WS17 was further found to survive and degrade the compound in the presence of a mixed bacterial culture from a sewage

treatment lagoon.

FUTURE PROSPECTS

The studies conducted during this research with mixed phototrophic, dehalogenating cultures, and various strains of *R. palustris*, should be considered as the first step toward:

(i) Application of phototrophs for bioremediation processes under anaerobic environments. These processes could be put to better use with further studies on the substrate range determination for phototrophs. Such substrate range studies can also focus light on structure-activity (biodegradability) relationships of various compounds. A group of researchers headed by Dr. B. J. Van der Woude, Department of Microbiology, University of Groningen, Netherlands, involved in studying the environmental factors influencing the capacities of microorganisms to transform xenobiotics has requested for *R. palustris* WS17. We have already supplied them, the 3-chlorobenzoate degrading *R. palustris* strain WS17.

(ii) The use of phototrophs in wastewater treatment systems for various industrial effluents. I have observed the presence of photosynthetic bacteria as copious red mats in wastewaters held in lagoons which were open to

sunlight and were receiving high loadings of organic matter, such as pulp and paper mill effluents and food manufacturing waste streams. The bacterial growth was persistent even after the lagoon was aerated. The existence of large blooms of purple photosynthetic bacteria was also observed by Federle (1989) in detergent waste and by Mitsui et al. (1985) in food processing waste. In these environments complex mixtures of aromatic substrates may be important substrates for phototrophic growth. *R. palustris* may be responsible in some cases for the removal of halogenated aromatics from these waste waters.

(iii) The use of phototrophs as a simpler anaerobic system (as compared to the methanogens) for research into the biochemical mechanisms of reductive dehalogenation and several other processes of anaerobic metabolism. Methanogens being strict anaerobes require special culture techniques and equipments to work with. However, phototrophs need no special care except for the source of light and use of tightly closing culture flasks or bottles. During this research the phototrophic isolate *R. palustris* WS17 was found to degrade 3-chlorobenzoate at the maximum rate of 0.113 mM/day under optimum conditions. Whereas *D. tiedje*, the dehalogenating member from a methanogenic consortium was reported to

dehalogenate the compound at the maximum rate of 0.0646 mM/day (Shelton and Tiedje 1984). This indicates higher efficiency of dehalogenation for WS17 which is a desirable character for bioremediation processes.

(iv) Further verification and filling in of gaps in the pathway as proposed in figure 5.3, through enzymatic studies, and;

(v) Characterization of the benzoate/chlorobenzoate reductive pathway genes in the chromosome of *R. palustris*. The small plasmid found in most *R. palustris* strains and their kanamycin sensitivity may be exploited for Tn5 mutagenesis and complementation studies.

The nature of the potential research projects arising out of the work described in this Thesis indicates that the future of this field is indeed bright.

APPENDIX - II

R. palustris WS17 CELL LYSIS

1. Harvest cells and wash once with 10 mM phosphate buffer (pH 7.5) or TEA-PO₄ (pH 7.5).
2. Resuspend the cell pellet in approximately 2 volumes of the above buffers.
3. Freeze the cells at this point, at -20⁰C, to store them and to weaken cell walls.
4. Stir the cells on the magnetic stirrer with the pH electrode in place, and maintain the pH at 7.5 during the following steps:
5. Add solid lysozyme to a final concentration of 1 mg/ml and add 0.5 M EDTA (pH 8) to a final concentration of 5 mM.
6. Stir at room temperature, keeping the pH at 7.5, for 30 min.
7. Freeze cells at this point, at -20⁰C, to weaken cell walls.
8. Thaw the cells and sonicate at full output with the Parge probe, 50% duty cycle setting on Vibra Cell sonicator for 3 minutes on ice. Check the temperature occasionally and do not let it go above 10⁰C. Repeat the sonication when the cells have returned to 0⁰C on ice.

9. Centrifuge at 10,000 rpm, at 4°C for 20 minutes.
10. Nucleic acids may be removed by treating the supernatant with 10 mM MgCl₂, 0.01 mg/ml DNase and 0.01 mg/ml RNase.
11. This lysate can be further used for cell free extract studies or for extraction of intracellular metabolic products.

APPENDIX - III

The figure shows typical variation of pH in *R. palustris* WS17 cultures over the total period of incubation. For medium P with benzoate alone, mean value was 7.034 with standard deviation of ± 0.034 which equals 0.48% coefficient of variation. For medium P with benzoate and 3-chlorobenzoate, mean value was 7.005 with standard deviation of ± 0.039 which equals 0.56% coefficient of variation.

APPENDIX - IV

The figures show Lineweaver Burk Plots for benzoate and 3-chlorobenzoate metabolism in *R. palustris* WS17. Slopes of the lines present values for $K_s/\mu_{\max}$ and Y-intercept indicates $1/\mu_{\max}$. $\mu_{\max}$ and K_s are calculated from these values. For benzoate degradation in WS17 $\mu_{\max}=1.658$ mM/Day and $K_s=0.077$ mM. Respective values for 3-chlorobenzoate degradation are 0.279 mM/Day and 0.1117 mM.

FOR BOTH FIGURES :
 v : RATE OF SUBSTRATE
 UTILIZATION.
 s : SUBSTRATE CONCENTRATION

LINEWEAVER BURS PLOT FOR
 KINETICS OF BENZOATE DEGRADATION
 IN WS17

LINEWEAVER-BURK PLOT FOR
 3-CHLOROBENZOATE DEGRADATION
 BY WS17

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