ISOLATION AND PHYSIOLOGICAL CHARACTERIZATION OF TWO CHLOROBENZOIC ACID DEGRADING BACTERIA FROM POLYCHLORINATED BIPHENYL CONTAMINATED SOILS

by

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ABSTRACT

Ph.D. Carlos B. Miguez Microbiology

Isolation and physiological characterization of two chlorobenzoic acid degrading bacteria from polychlorinated biphenyl contaminated soil

Two strains of *Alcaligenes denitrificans*, designated BRI 3010 and BRI 6011, were isolated from polychlorinated biphenyl (PCB) contaminated soil using 2,5-dichlorobenzoic acid (2,5-DCBA) and 2,4-DCBA, respectively, as sole carbon and energy sources. Both strains degraded 2-chlorobenzoic acid (2-CBA), 2,3-DCBA, and 2,5-DCBA. BRI 6011 alone degraded 2,4-DCBA. Metabolism of the chlorinated substrates resulted in the stoichiometric release of chloride, and degradation proceeded by intradiol cleavage of the aromatic ring. Growth of both strains on dichlorobenzoic acids induced pyrocatechase activities having catechol (catechol 1,2-dioxygenase) and chlorocatechols (chlorocatechol 1,2-dioxygenase) as substrates. Growth on 2-CBA and benzoic acid induced a pyrocatechase activity (catechol 1,2-dioxygenase) directed against catechol only.

The chlorocatechol 1,2-dioxygenase from BRI 6011 was purified, characterized, and compared with the chlorocatechol 1,2-dioxygenase from *Pseudomonas* sp. B13 and *P. putida*, organisms limited with respect to their CBA degradative versatility. These enzymes appear to be very similar based on biochemical and genetic data and possess sufficient broad substrate specificity to accommodate a wide range of chlorinated catechols, hence the increased versatility for chlorobenzoic acid degradation of *A. denitrificans* can not be attributed to a more specialized chlorocatechol 1,2-dioxygenase.

Uptake of benzoic acid by BRI 3010 and BRI 6011 was inducible, exhibited saturation kinetics and the substrate was accumulated intracellularly against a concentration gradient by a factor of 8 and 10, respectively, indicative of active transport. Uptake of 2,4-DCBA by BRI 6011 was constitutive and saturation kinetics were not observed, suggesting passive diffusion of 2,4-DCBA and other CBAs into the cell down a concentration gradient.

Based on oxygen uptake experiments with whole cells, benzoic acid dioxygenase and chlorobenzoic acid dioxygenase activity was induced by benzoic acid and *ortho*-substituted chlorobenzoic acids, respectively. Since 2,4-DCBA diffuses across the membrane and the expected catecholic intermediates of 2,4-DCBA metabolism are metabolizable by BRI 3010, this suggests that the major difference between BRI 3010 and BRI 6011 might be the inability of the chlorobenzoic acid dioxygenase in BRI 3010 to recognize 2,4-DCBA as a substrate.

RESUME

Ph.D. Carlos B. Miguez Microbiologie

Isolement et caractérisation de deux microorganismes dégradant l'acide chlorobenzoïque, à partir de sol contaminé aux biphényles polychlorés.

Deux souches d'*Alcaligenes denitrificans*, désignées BRI 3010 et BRI 6011, ont été isolé de sol contaminé aux biphényles polychlorés (BPC) en utilisant respectivement l'acide 2,5-dichlorobenzoïque (2,5-DCBA) et le 2,4-DCBA , comme seule source de carbone et d'énergie. Les deux souches dégradaient l'acide 2-chlorobenzoïque (2-CBA), le 2,3-DCBA et le 2,5-DCBA. Seulement BRI 6011 dégradait le 2,4-DCBA. Le métabolisme des substrats organochlorés résultait à un largage stoechiométrique du chlore, et la dégradation démontrait un clivage intradiole du noyau aromatique. La croissance des deux souches sur les acides dichlorobenzoïque induisait des activités pyrocatéchase possédant le catéchol (catéchol 1,2-dioxygénase) et les chlorocatéchols (chlorocatéchol 1,2-dioxygénase) comme substrats. La croissance sur le 2-CBA et l'acide benzoïque induisait seulement l'activité pyrocatéchase (catéchol 1,2-dioxygénase) dirigée contre le catéchol.

Le chlorocatéchol 1,2-dioxygénase de BRI 6011 a été purifié, caractérisé et comparé avec le chlorocatéchol 1,2-dioxygénase de *Pseudomonas* sp B13 et *P. putida* , organismes qui sont limités dans leur versatilité de dégradation des CBA. Ces enzymes semblent être très similaire au point de vue biochimique et génétique et ils possèdent une large spécificité, suffisant pour accommoder un large spectre de chlorocatéchols comme substrats, par conséquent l' augmentation de la versatilité de la dégradation de l'acide

chlorobenzoïque par *A. denitrificans* peut ne pas être attribué à un chlorocatéchol 1,2-dioxygénase plus spécialisé.

L'utilisation de l'acide benzoïque par BRI 3010 et BRI 6011 était inductible, démontrait une cinétique de saturation et le substrat était accumulé intracellulairement contre un gradient de concentration d'un facteur de 8 à 10 respectivement, indicatif du transport actif. L'utilisation du 2,4-DCBA par BRI 6011 était constitutive et la cinétique de saturation n'a pas été observé, suggérant une diffusion passive du 2,4-DCBA et des autres CBAs à l'intérieur de la cellule engendrer par le gradient de concentration.

Basées sur les expériences d'utilisation de l'oxygène avec des cellules entières, l'activité de l'acide benzoïque dioxygénase et de l'acide chlorobenzoïque dioxygénase étaient induites respectivement par l'acide benzoïque et les acides chlorobenzoïques *ortho*-substitués . Puisque le 2,4-DCBA diffuse à travers la membrane et les intermédiaires catécholiques attendus du métabolisme du 2,4-DCBA pouvaient être métabolisés par BRI 3010, ceci suggère que la différence majeure entre BRI 3010 et BRI 6011 devrait être l'incapacité de l'acide chlorobenzoïque dioxygénase de BRI 3010 de reconnaître le 2,4-DCBA comme substrat.

To Rosa, Daniel, Kayla, Pilar, Felisa, and Pedro

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I wish to express my indebtedness to Dr. Charles W. Greer, my co-supervisor, for his friendship, moral support, and devoted scientific guidance. His enthusiasm in the project and the countless therapy sessions, will not be easily forgotten.

Personal thanks and appreciation are sent to my parents Pedro and Felisa, and my sister Pilar, for their unyielding moral support.

I wish to especially thank my wife Rosa whose love and understanding has made the fruition of this endeavour a reality, and my son, Daniel, for keeping me anchored to the finer things life has to offer and for guiding me through his wonderful world of innocence.

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Two chlorobenzoic acid degrading microorganisms, *Alcaligenes denitrificans* designated BRI 3010 and BRI 6011 were isolated from an environmental soil sample contaminated with polychlorinated biphenyl (Aroclor 1254, 800 ppm) by enrichment culture using 3 mM 2,5-DCBA and 3 mM 2,4-DCBA as sole carbon and energy sources, respectively. These two bacteria were physiologically characterized.

2. *Alcaligenes denitrificans* strain BRI 3010 and BRI 6011 degraded the notoriously recalcitrant *ortho*-substituted chlorobenzoic acids, 2-CBA, 2,3-DCBA, and 2,5-DCBA. BRI 6011 alone degraded 2,4-DCBA. Both strains also degraded benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid.

3. The degradation of chlorobenzoic acids by both strains proceeded by intradiol cleavage of the aromatic ring. The strains possessed both catechol 1,2 dioxygenase and chlorocatechol 1,2-dioxygenase activity.

   a. BRI 3010 and BRI 6011 grown on benzoic acid and 2-CBA induced pyrocatechase activity directed against catechol, hence the presence of a catechol 1,2-dioxygenase.

   b. BRI 6011 grown on 2,4-DCBA and 2,5-DCBA, and BRI 3010 grown on 2,5-DCBA induced pyrocatechase activity directed against mono- and dichlorocatechols, hence, the presence of a chlorocatechol 1,2-dioxygenase.

4. The chlorocatechol 1,2-dioxygenase from *Alcaligenes denitrificans* BRI 6011 was purified and characterized. Catechol, 3-chlorocatechol (3-CC), 4-CC, 3,4-dichlorocatechol (3,4-DCC), 3,5-DCC, 3,6-DCC, 3-methylcatechol (3-MC), and 4-MC served as substrates for the enzyme. The V_{max} for the dichlorocatechols were similar, while those for the mono-chlorinated and methylated catechols were higher. The K_m for all the chlorinated catechols were typically below 1 μM, while those for catechol and the methylated catechols were above 10 μM. This enzyme was virtually identical to the chlorocatechol 1,2-dioxygenase from *Pseudomonas* sp. B13 and *Pseudomonas putida* (pAC27) on the basis of biochemical and genetic studies, although these three bacteria were isolated from geographically distinct areas. *Pseudomonas sp.* B13 and *P. putida* were isolated from sewage samples in Germany and in the U.S., respectively, whereas *A. denitrificans* was isolated from soil samples from Smith Falls, Ontario, Canada.

5. The uptake mechanism of aromatic and chloro-aromatic compounds by BRI 3010 and BRI 6011 was determined

   a. Uptake of benzoic acid into BRI 3010, displayed monophasic saturation kinetics and the substrate was concentrated inside the cells against a gradient, suggesting an active uptake mechanism for benzoic acid.

   b. Uptake of benzoic acid into BRI 6011, displayed biphasic saturation kinetics indicative of two saturable uptake systems for benzoic acid. Benzoic acid was concentrated inside the cells against a gradient, suggesting an active uptake mechanism for benzoic acid.

c. Uptake of 2,4-DCBA into BRI 6011 did not display saturation kinetics, and the substrate was not concentrated inside the cells against a gradient, suggesting the passive diffusion of 2,4-DCBA into the cell down a concentration gradient and maintained by the metabolism of the entering compound.

6. BRI 3010 and BRI 6011 possessed both benzoic acid dioxygenase and chlorobenzoic acid dioxygenase activities. Growth on CBAs induced chlorobenzoic acid dioxygenase activity which catalyzed the oxygenation of the *ortho*-substituted CBAs utilized by BRI 3010 and BRI 6011. Dioxygenation of 2,4-DCBA by BRI 3010 was not observed.

7. A sequential pattern of CBA utilization was observed in BRI 6011 when grown in mixed CBA substrate conditions. The presence of a preferred CBA substrate retarded the utilization of other CBA substrates in the mixture. This sequential pattern of substrate utilization can markedly influence the efficacy of bioremediation of environments typically contaminated with heterogeneous organic mixtures.

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MANUSCRIPTS AND AUTHORSHIP

The following text, from the "Guidlelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, must be "cited in full in the introductory sections of any thesis to which it applies":

The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be providied in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationary and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary material is almost always necessary.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review.

Contributions of co-authors to manuscripts for publication

Sections 3 and 4 were drawn from manuscripts co-authored by C. B. Miguez, J. M. Ingram, and C. W. Greer. These manuscripts were published as follows:

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Drs. J. M. Ingram and C. W. Greer, my thesis co-supervisors, acted in a supervisory capacity throughout this study and reviewed the manuscripts before they were submitted for publication. Dr. R. A. MacLeod co-supervised and reviewed the work presented in section 5 of this thesis.

Section 1

Introduction

The accidental or planned release of industrially produced aromatic halogenated compounds into the environment has resulted in considerable global pollution and has heightened public health concerns. Such compounds include biocides (2,4-dichlorophenoxyacetic acid [2,4-D], 2,4,5-T, chlorophenols, 2-methoxy-3,6-dichlorobenzoic acid [dicamba], 2,5-dichloro-3-aminobenzoic acid, 2,3,6-trichlorobenzoic acid, 2,6-dichlorobenzonitril, chlorobenzenes) and the polychlorinated biphenyls (PCBs) extensively used as constituents of insulators in electrical transformers and lubricating oils.

The notoriously low rates of microbial degradation of these compounds in nature is due to several factors. Ecosystems are rarely contaminated with a single chloroaromatic compound, but rather with heterogeneous mixtures of these compounds with varying concentrations and toxicity. These compounds are alien to existing enzyme systems within the indigenous microbial population in nutrient poor environments and consequently are not metabolized. However, with time microbial populations may adapt to the presence of these compounds and degrade certain of the mixed chlorinated aromatic compounds. The process of adaptation to growth on one or several of the contaminating compounds is usually slow but can occur by induction of specific enzymes within members of the microbial population which possess the appropriate degradative pathways. This usually results in an increase in numbers of a sub-population with the pertinent degradative capabilities. The acquisition of new genes governing the synthesis of new enzymes with broader substrate specificity by mutational events, recombination, transformation or genetic exchange within members of the microbial population, can also occur.

Assuming that one or all of the above mentioned processes occurs, degradative processes can also be limited by varying physicochemical conditions including temperature, pH, redox potential, salinity, oxygen tension, and substrate solubility (van der Meer et al.1992).

The microbial degradation of PCBs in nature requires the fruitful interaction between biphenyl utilizing bacteria which can fortuitously transform PCBs to chlorobenzoic acids, and chlorobenzoic acid degrading bacteria (Kilpi et al. 1988; Liu 1980, Clark et al. 1979; Ahmed and Focht 1973; Brunner et al. 1985; Adriaens et al. 1991; Furukawa et al. 1978; Kohler et al. 1988; Nies and Vogel 1990; Furukawa et al. 1979). However, incompatibility of degradative pathways within an organism or within the microbial population can also occur, where a metabolite of a degradative pathway inhibits the catalytic activity of an enzyme in another pathway (Rojo et al. 1987; Hartmann et al.1979; Haigler et al. 1992; Sondossi et al. 1992). For example, 3-chlorocatechol a potential intermediate in the degradation of certain chlorobenzoic acids inhibits the *meta*-cleavage enzyme of the biphenyl-chlorobiphenyl degradative pathway (Sondossi et al. 1992; Adams et al. 1992). Apart from the chlorobenzoic acids introduced into the environment by industrial and agricultural practices, chlorobenzoic acids accumulate in nature due to the failure of the above mentioned interaction between biphenyl and chlorobenzoic acid degraders and pathway incompatibility. It is principally for this reason that the work presented in this thesis was initiated. This study began as a part of a research project aimed at isolating and characterizing cultures capable of degrading chlorobenzoic acid, specifically 2,5-DCBA, from PCB contaminated soils. The reason for the emphasis on 2,5-DCBA degradative capabilities was three-fold. Firstly, the degradation of chlorobiphenyls is initiated by 2,3-dioxygenation attack on the phenyl ring with the least amount of chlorine substitution. The

presence of a chlorine atom in either the 2 or 3 position on either ring inhibits such dioxygenation. If however, the chlorinated biphenyl does have free 2,3 sites on one of the rings, 2,3-dioxygenation will occur. Consequently, the dichlorobenzoic acids most likely to be formed are those with 2,5- or 3,5-substitution patterns (Hernandez et al. 1991). Therefore, microorganisms capable of degrading these chlorinated benzoic acids are essential for the complete degradation of certain PCBs. Secondly, microbial reductive dechlorination of Aroclor 1242, which is the PCB mixture most frequently encountered as a contaminant in nature, occurred primarily from the *meta*- and *para*-position thereby accumulating *ortho*-substituted chlorinated biphenyls (Quensen et al. 1988). Thirdly, *ortho*-substituted mono- and dichlorobenzoic acids are more refractory than other isomers to biodegradation. Many microorganisms capable of degrading 3-chlorobenzoic acid (3-CBA) have been reported (Chatterjee et al. 1981; Dorn et al. 1974; Horvath and Alexander 1970; Haller and Finn 1979; Reber and Thierbach 1980). Less abundant are 4-CBA degrading microorganisms (Keil et al. 1981; Klages and Lingens 1979; Marks et al. 1984; van den Tweel et al. 1987). However, at the onset of this research project, few natural isolates capable of degrading 2-CBA had been reported (Taylor et al. 1979; Zaitsev and Karasevich 1984; Baggi 1985). Equally uncommon was the degradation of 2,5-DCBA by axenic cultures (Baggi 1985). Recently, natural isolates capable of degrading 2-CBA (Sylvestre et al. 1989; Engesser and Schulte 1989; Fetzner et al. 1989a, 1989b), and 2,5-DCBA have been reported (Hickey and Focht 1990; Hernandez et al. 1991). The resistance of 2-CBA and 2,5-DCBA to dioxygenation by the benzoic acid 1,2-dioxygenase is due to steric hindrance and the effect of chlorine electron density at the *ortho*-position of the benzene ring (Reineke and Knackmuss 1978a). However, these organisms have surmounted this biocatalytic constraint by the synthesis of a

novel enzyme, chlorobenzoic acid 1,2-dioxygenase, which as a result of the dioxygenation event, dehalogenation and decarboxylation occur simultaneously, forming catechol and 4-chlorocatechol from 2-CBA and 2,5-DCBA, respectively (Hernandez et al. 1991; Sylvestre et al. 1989; Fetzner et al. 1992).

This thesis describes the isolation and characterization of two isolates, *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011, unique *vis a vis* the range of chlorobenzoic acids which serve as growth substrates. Both organisms can utilize 2-CBA, 2,3- , and 2,5-DCBA, as sole carbon and energy sources. BRI 6011 can also utilize 2,4-DCBA. In order to ascertain the factors responsible for this apparent enhanced chlorobenzoic acid substrate specificity we investigated (i) the range of aromatic substrates utilized by both organisms, (ii) the mechanism(s) of aromatic ring cleavage, (iii) the purification and characterization of a chlorocatechol 1,2-dioxygenase from BRI 6011, (iv) the uptake mechanism of aromatic and chloroaromatic substrates by BRI 3010 and BRI 6011, (v) the mechanism of initial substrate dioxygenation, and (vi) the pattern of mixed substrate utilization in order to address the efficacy of biodegradation in the environment.

Section 2

Literature Review

2.1 Polychlorinated biphenyls (PCBs)

PCBs have been manufactured commercially since 1929, with production estimates in 1988 at about two million tonnes. Although their use has been legislated and banned in the United States since 1971 and in Europe since the late nineteen seventies, their use and consequently their release into the environment is still occurring (Kilpi et al. 1988). As a consequence of their recalcitrance to chemical and biological degradation, PCBs are regarded as global contaminants (Chaudhry and Chapalamadugu 1991). PCBs are synthesized by direct chlorination of biphenyl with chlorine gas. Depending on the extent of chlorination, the polychlorinated biphenyl molecule can be one of 209 discrete compounds having the formula $C_{12}H_{10-n}Cl_n$, where n= 1-10. The entire set of PCBs are also referred to as congeners which are analogues varying in the number and arrangement of chlorine substituents. One group of commercial compounds are referred to as Aroclor, a registered trade-mark of Monsanto (Erickson 1986) and these mixtures can contain up to 100 different congeners. For example, Aroclor 1254 is a complex mixture of polychlorinated biphenyls, where the first two digits (12) represent the number of carbon atoms in the biphenyl skeleton and the two last digits (54) represent the percentage of chlorination. These compounds are essentially water insoluble, non-polar, and lipid soluble. PCBs have been extensively used in electronic equipment as dielectric fluids, in capacitors and transformers, heat transfer fluids, lubricating oils, and as additives in plastics, paints, and pesticides (Erickson 1986). Slow rates of abiotic degradation of these compounds via photolysis has been shown to occur in the environment, however the bulk of PCB contaminants in nature

are not exposed to natural light (Hawari et al. 1992a; 1992b). The outcome of the microbial degradation of PCBs is the raison d'être for the work presented here and merits a review of the microbial degradation patterns and consequences of such actions.

2.1.1 Biphenyl degradation

Biphenyl (BP) is a phenyl-substituted benzene. Mechanisms of BP degradation have received close scrutiny since it was hoped they would serve as a model in the study of PCB degradation (Smith 1990). The pathway of BP degradation by most bacteria is shown in figure 2.1. The initial attack on the BP molecule proceeds by the incorporation of two atoms of molecular oxygen at the 2,3 position of either benzene ring, catalyzed by a biphenyl dioxygenase forming 2,3-dihydro-2,3-dihydroxybiphenyl. Sequentially, a dihydrodiol dehydrogenase converts the former compound to 2,3-dihydroxybiphenyl which is a catecholic compound. The dihydroxylated ring is then cleaved between carbon 1 and 2 by a 2,3-dihydroxybiphenyl 1,2-dioxygenase to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-diencate (Catelani et al. 1973; Gibson et al. 1973; Catelani et al. 1974; Smith and Ratledge 1989). Further metabolism of the ring fission-product proceeds via 2-oxo-penta-4-enoate and benzoic acid (Smith and Ratledge 1989). The compound 2-oxo-penta-4-enoate is readily metabolized, whereas, the fate of benzoic acid depends largely on the degradative capabilities of the BP-degrading microorganism. In *Pseudomonas* sp. NCIB 10643, benzoic acid was a dead-end metabolite. More commonly, the benzoic acid is degraded via oxidative-decarboxylation to catechol. Again, depending on the microorganism, catechol can be *meta*- or *ortho*-cleaved (Furukawa and Chakrabarty 1982; Smith and Ratledge 1989). *Meta* and *ortho* -cleavage mechanisms are described in section 2.2.2.

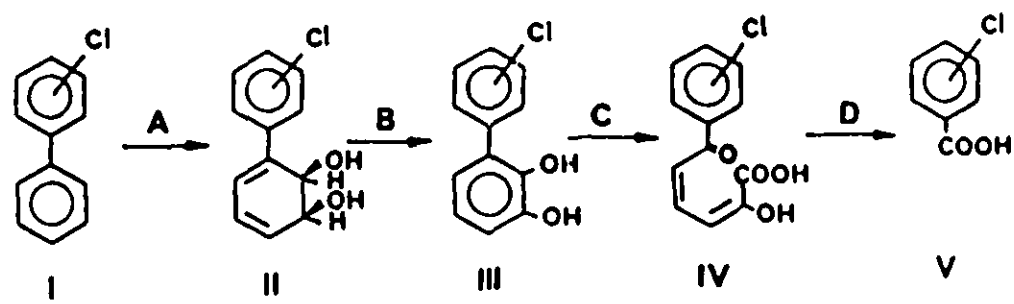


Figure 2.1

The pathway for the aerobic degradation of biphenyl and chlorinated biphenyls.

Compounds: I, (*chlorinated*) biphenyl; II, (*chlorinated*) 2,3-dihydroxy-4-phenylhexa-2,4-diene (dihydrodiol compound); III, (*chlorinated*) 2,3-dihydroxybiphenyl; IV, (*chlorinated*) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage compound); V, (*chlorinated*) benzoic acid.

Enzymes: A, biphenyl dioxygenase; B, dihydrodiol dehydrogenase; C, 2,3-dihydroxybiphenyl 1,2-dioxygenase (*meta*-cleaving enzyme); D, *meta*-cleavage compound hydrolase. Adapted from Furukawa et al. (1989).

2.1.2 Polychlorinated biphenyl degradation

The aerobic degradation of chlorinated biphenyls has been observed in both Gram-negative and Gram-positive bacteria consisting mostly of members of the genera *Pseudomonas*, *Alcaligenes*, *Arthrobacter*, and *Acinetobacter* (Commandeur and Parsons, 1990). Although the degradation of single or mixed chlorinated biphenyls has been observed based on the disappearance of substrate from the growth medium or by mineralization (formation of CO_2), generally chlorinated benzoic acids are formed as dead-end metabolites (Ahmed and Focht 1973; Bedard et al. 1986; Furukawa et al. 1979; Furukawa and Chakrabarty 1982; Furukawa et al. 1989; Yagi and Sudo 1980). Furukawa et al. (1979) described the PCB degradation pathway sequence in *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6. Interestingly, the chemical nature of the intermediates in the degradative pathway of biphenyl and chlorinated biphenyls are identical except for the chlorine substituents (Fig 2.1).

Brunner et al. (1985) showed that the addition of *Acinetobacter* sp. strain P6 together with biphenyl, enhanced the mineralization of added [^{14}C] Aroclor 1242 in soil, whereas the addition of strain P6 alone did not. Biphenyl supported the growth of strain P6, with subsequent cometabolic oxygenase attack on the PCBs. The halogenated products formed as a consequence of PCB co-metabolism, chlorobenzoic acids, were subsequently metabolized by endogenous soil microflora. Kohler et al. (1988) showed that *Acinetobacter* sp. P6 and *Arthrobacter* sp. strain B1B cometabolized PCBs by transforming a non-growth substrate Aroclor 1254, only in the presence of the growth substrate, biphenyl. This co-metabolism of PCBs appears not to be an uncommon phenomenon (Ahmed and Focht 1973; Brown et al. 1987; Furukawa and Chakrabaty 1982; Furukawa et al. 1978; Kilpi et al. 1988; Parsons et al. 1988; Sondossi et al., 1992; Havel and Reineke, 1991). Cometaboilsm of PCBs

occurs for the main reason that biphenyl and chlorinated biphenyls are degraded through a common pathway (Ahmad et al. 1990; Furukawa et al. 1983; Parsons et al. 1983, 1988; Reineke and Knackmuss, 1988; Sondossi et al. 1991, 1992). However, there have been several reports of notable differences in the congener selectivity patterns and ranges of activity of various PCB-degrading bacteria (Bedard and Haberl 1990; Furukawa and Matsumura 1976, Furukawa et al. 1979), which suggests a broadening of the specificities of the BP pathway enzymes, but complete mineralization of a PCB molecule by axenic natural isolates has not been described. This has only been achieved by mixed cultures (co-cultures) consisting of a CBA degrader in conjunction with a BP-CBP degrader. Such associations have only been reported to degrade congeners that produce 4-CBA as the end product of the BP-CBP degradation pathway by the CBP-degrading member (Adriaens et al. 1989; Pettigrew et al. 1990; Sylvestre et al. 1985). Sondossi et al. (1992) suggested that the lack of reports on co-cultures degrading CBP molecules which result in the formation of CBAs other than 4-CBA may be due to pathway incompatibility. Similarly to other BP-degraders, *Pseudomonas testosteroni* B-356 degraded BP via benzoic acid, which was subsequently metabolized by an unknown mechanism. They observed that biphenyl-grown resting cell suspensions incubated with CBAs, fortuitously transformed CBAs by non-specified oxygenases to chloro-1,2-dihydro-1,2-dihydroxybenzoic acids, catechol and chlorinated catechols. These metabolites were absent in benzoic acid-grown resting cell suspensions incubated with the same CBAs. The chlorocatechols formed as a consequence of the non-specified oxygenation of the CBAs by BP-grown cells, inhibited BP degradation. Sondossi et al. (1992) observed that 3-chlorocatechol (3-CC) at a concentration of 500 pmol per ml of reaction mixture inhibited 99% of the activity of the 2,3-dihydroxybiphenyl 1,2-dioxygenase, the

meta-cleavage enzyme of the BP pathway. They stipulated that the nature of the inhibition is analogous to the inhibition of catechol 2,3-dioxygenase (*meta*-cleavage enzyme) by 3-CC (see section 2.2.2.1). Catalytic attack of 3-CC by the *meta*-cleavage enzyme, yields 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid which is a strong inhibitor of the enzyme (Bartels et al. 1984; Klecka and Gibson 1981; Reineke and Knackmuss 1980). Catechol and 4-CC were inferior inhibitors of the 2,3-dihydroxybiphenyl 1,2-dioxygenase, requiring 450 and 90 nmol/ml, respectively, to inhibit the enzyme activity by 99%. These observations made by Sondossi and co-workers (1992) may help explain the phenomenon previously mentioned, where degradation of CBP molecules by co-cultures has only been reported when 4-CBA is the chlorobenzoic acid intermediate. The formation and subsequent metabolism of any other chlorinated benzoic acids via 3-CC will thereby inhibit BP ring cleavage and consequently degradation of BP and CBP. The co-cultures capable of degrading 4-CBP, form 4-CBA as the CBA intermediate which is subsequently metabolized via 4-hydroxybenzoic acid (4-HBA) and protocatechuic acid (3,4-DHBA) thus avoiding the inhibitory effect of chlorinated catechols. However, due to the extremely narrow specificity of the 4-CBA dechlorinating enzyme, such a co-culture is limited to the degradation of 4-CBP, since other congeners such as 3-CBP or 3,5-DCBP will yield isomeric forms of chlorinated benzoic acids other than 4-CBA (Klages and Lingens 1979; Marks et al. 1984a, 1984b; Ruisinger et al. 1976; van den Tweel et al. 1986). Two strains, *Alcaligenes* sp and *Acinetobacter* sp. have been reported to mineralize 4-CBP completely through 4-CBA (Shields et al. 1985).

A similar observation of incompatibility between the BP and BA pathways was reported by Adams et al. (1992). They constructed a 3-CBP-utilizing recombinant, *Pseudomonas* sp. strain CB15, from an intergenic mating of *Pseudomonas* sp. strain HF1, which grows on 3-CBA, and from *Acinetobacter*

sp. strain P6 which grows on BP. The recombinant strain dissimilates benzoic acid and 3-CBA by intradiol cleavage (*ortho*) of the aromatic ring (catechol). Although the recombinant strain utilized 3-CBP, growth was very poor. This was attributed to three possible inhibitions from catecholic intermediates. Firstly, the *meta*-cleavage enzyme, 2,3-dihydroxybiphenyl 1,2-dioxygenase, was inhibited by 3-chloro-2',3'-dihydroxybiphenyl, a metabolite in the degradation of 3-CBP. Secondly, this enzyme is strongly inhibited by 3-CC, which is an intermediate in the degradation of 3-CBA (3-CBA is the end-product of the CBP-pathway). Thirdly, the *ortho*-cleavage of 3-CC was competitively inhibited by 2,3-dihydroxybiphenyl and presumably also by its chlorinated analogue (Fig. 2.2).

The slow and incomplete mineralization of 3-CBP by a hybrid strain *Pseudomonas* sp. strain BN210, obtained from the mating between *Pseudomonas putida* strain BN10 and *Pseudomonas* sp. strain B13, is presumably due to the same factors described above (Mokross et al. 1990). *Pseudomonas* sp. B13 dissimilated 3-CBA through 3-CC which as shown by Sondossi et al. (1992) and Adams et al. (1992) inhibits the BP-CBP pathways.

Although several recombinant strains capable of growth on chlorinated biphenyls have been obtained (examples cited), the growth rate, mineralization rate, and the range of CBP substrates utilized, is poor. Lack of compartmentalization of the inhibitory metabolites within the cell may be a definite limiting factor. Until a means is found where this constraint imposed by the incompatibilities between the BP-CBP and CBA pathways is alleviated, the search for CBA degraders with enhanced substrate versatility and degradative efficiency, will be on-going.

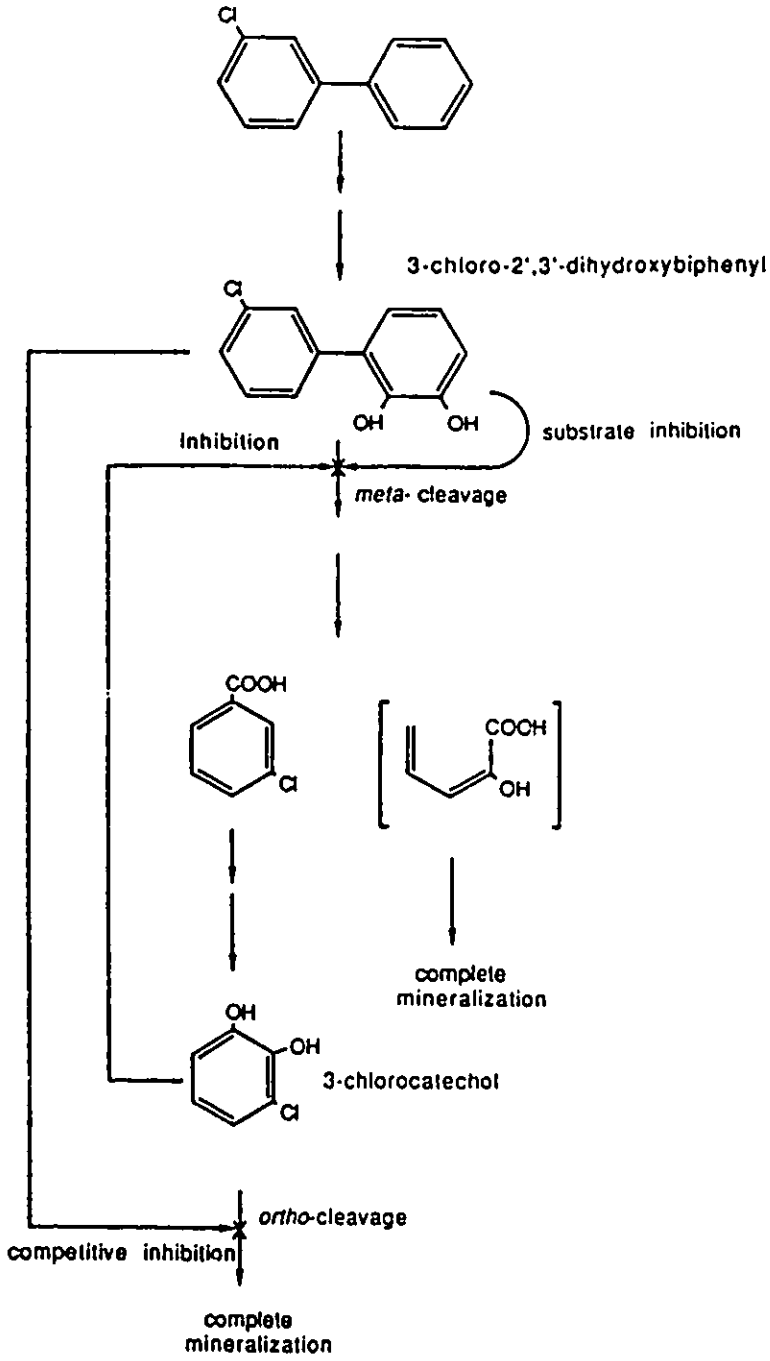


Figure 2.2

Metabolism of 3-chlorobiphenyl and product inhibition in *Pseudomonas* sp. strain CB15. Taken from Adams et al. (1992).

2.1.3. Anaerobic degradation of PCBs

In comparison to the mechanisms of aerobic metabolism of PCBs, less is known concerning the anaerobic transformation of these molecules. However, PCBs have been shown to be reductively dechlorinated by anaerobic microorganisms in PCB-contaminated sediments (Alder et al. 1990; Nies and Vogel 1990; Quensen et al. 1990; Van Dort and Bedard 1991; Ye et al. 1992; Morris et al. 1992). Reductive dechlorination is the process whereby the chlorine substituent is removed from a molecule with the simultaneous addition of electrons to the molecule. The process is known as hydrogenolysis where the chlorine is replaced with a hydrogen atom and the process requires an electron donor (reductant). Unlike the aerobic metabolism of PCBs, where only low chlorinated congeners are transformed, anaerobic reductive dechlorination converts highly chlorinated PCBs into lesser chlorinated mono- and dichlorobiphenyls, which can subsequently be degraded more readily by aerobic microorganisms (Brown et al. 1987; Quensen et al. 1988, 1990). Enzyme specificity has also been observed in reductive dehalogenation. Dehalogenation is most commonly observed at the *meta* and *para* positions, although *ortho* dehalogenation has recently been shown to occur (Van Dort and Bedard 1991). Consequently the major products of PCB anaerobic degradations are *ortho*-substituted mono- and dichlorobiphenyls (Ye et al. 1992; Mohn and Tiedje 1992)

2.1.4. Genetic location and arrangement of BP-CBP genes

Genes encoding BP-CBP catabolism have been located on plasmids (Furukawa and Chakrabarty 1982; Shields et al. 1985), and on the chromosome (Furukawa et al. 1989: Furukawa and Miyazaki 1986; Khan et al. 1988; Khan and Walia 1990). Plasmid pSS50 (35 X 10^6 dalton) extracted from

both *Alcaligenes* and *Acinetobacter* sp. was shown to mediate complete mineralization of 4-CBP. Furukawa and Chakrabarty (1982) isolated the pKF1 plasmid (53.7 X 10^6 daltons) from *Acinetobacter* sp. strain P6 and *Arthrobacter* sp. strain M5 which encodes for the degradation of 33 PCB isomers to the corresponding chlorinated benzoic acids, thereby making these two bacterial isolates the best PCB degraders yet described. Furukawa and co-workers (1989) studied the molecular relationship of chromosomal genes encoding BP/CBP catabolism in 15 different strains including *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Moraxella*, and *Arthrobacter* strains, all of which convert 4-CBP to 4-CBA. Three genes, *bphA* (encoding biphenyl dioxygenase), *bphB* (encoding dihydrodiol dehydrogenase), and *bphC* (encoding 2,3-dihydroxybiphenyl dioxygenase) are clustered in that order on the 9.4-kb *Xho*I DNA fragment forming an operon. The *bphD* gene, believed to be part of the same operon, encodes the hydrolyzing enzyme which converts 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid or its chlorinated analogue to benzoic acid or CBA and 2-oxo-penta-4-enoate (Hayase et al. 1990). It has been shown that the *bph* genes, including those that are plasmid encoded, are widely conserved and nearly identical at the nucleotide sequence level, with one major difference. The gene *bphC* from *P. paucimobilis* Q1, which encodes for 2,3-dihydroxybiphenyl dioxygenase, did not hybridize with any other *bphC* gene from all the BP-utilizing strains tested, nor was there any immunological homology. However, the enzymatic properties of 2,3-dihydroxybiphenyl dioxygenases were very similar in terms of their native and subunit molecular masses, cofactor requirements, and enzyme activities. Some conserved amino acid sequences were observed and speculated to function as binding domains for substrate, oxygen, and for the ferrous iron cofactor (Furukawa et al. 1989).

2.2 Aerobic degradation of chlorobenzoic acids

Aromatic compounds have been part of the environment for millions of years. Abiotic processes such as pyrolysis of organic compounds and biotic processes such as the synthesis and degradation of lignin have contributed to the formation and transformation of these compounds in nature. As a consequence of prolonged exposure to these substances, microorganisms have adapted to and developed appropriate degradative enzymes culminating in aromatic metabolism. Indeed, the high degree of conserved genes encoding for the enzymes of the biphenyl pathway among a large and varied array of bacterial species, is believed to have occurred as a consequence of such prolonged exposure. In contrast to the aromatic compounds, microorganisms have had a comparatively short time adapting to the presence of man-made chlorinated aromatic compounds. However, naturally occurring haloaromatic compounds exist in the environment , such as: (i) 2,4-dibromophenol synthesized by hemichordate worms (King 1986); (ii) aryl halides synthesized by red algae (Rhodophyta) (Mohn and Tiedje 1992); (iii) 2,4- and 2,6-dichlorophenol synthesized by *Penicillium* and lone star ticks, respectively (Siuda and DeBernardis 1973; and (iv) organochlorides as a result of the combustion of organic matter during forest fires (Mohn and Tiedje 1992).

Halogenation increases the recalcitrance of aromatic (arylhalides) compounds. Oxygenation of aromatic compounds is an electrophilic reaction. The presence of electron deficient substituents (halogens), lowers the electron density of the benzene nucleus thereby interfering with the process of oxygenation (Reineke and Knackmuss 1978a). The degradation of halogenated aromatic compounds is defined as the process by which the carbon skeleton of the aromatic compound is converted into intermediary metabolites and the organic halogen is returned to the mineral state (Reineke

and Knackmuss 1988). In recent years, considerable progress has been made in elucidating the different means by which micro-organisms achieve these two goals. Generally, several and very different pathways of aerobic chlorobenzoic degradation have been documented, all leading to the formation of central catecholic intermediates (catechol, chlorocatechols, and protocatechuic acid). The catecholic intermediates are then channeled either through an *ortho*- or a *meta*-cleavage pathway leading to the formation of tricarboxylic acid cycle intermediates.

2.2.1 Chlorobenzoic acid degradative pathways

Chlorobenzoic acid degradative pathways can be distinguished by whether or not dehalogenation occurs prior to the formation of the catecholic intermediate (i.e. dehalogenating and non-dehalogenating pathways). The dehalogenating peripheral pathways can be further subdivided by the mechanism by which dehalogenation occurs. In aerobic and facultative anaerobic microorganisms, dehalogenation without loss of aromaticity can occur by :

1 - Oxidative (oxygenolytic) dehalogenation in which the halogen is lost fortuitously (Reineke and Knackmuss 1988)

2 - Hydrolytic dehalogenation in which a halogen is specifically displaced by a hydroxyl group. This reaction can occur aerobically or under denitrifying conditions (Reineke and Knackmuss 1988).

3 - Reductive dehalogenation (hydrogenolysis) in which the removal of the halogen occurs concurrently with the addition of electrons (hydrogen atom) (Mohn and Tiedje 1992). This process has been observed predominantly under conditions of sulfate

reduction, nitrate reduction, photometabolism, and methanogenesis. Recently, reductive dehalogenation of 2,4-DCBA under aerobic conditions has been proposed (van den Tweel et al. 1987).

2.2.1.1 Conversion of chlorinated benzoic acids into chlorocatechols (non-dehalogenating pathway)

The conversion of chlorinated benzoic acid into chlorocatechols has been studied extensively with the 3-CBA-utilizing *Pseudomonas* sp. strain B13 and its derivative strains (Hartmann et al. 1979; Reineke and Knackmuss 1978a, 1978b, 1979; Reineke et al. 1982; Schmidt and Knackmuss 1980). *Pseudomonas* sp. B13, isolated from a sewage treatment plant in Göttingen, Germany, can utilize 3-CBA as a sole carbon source (Dorn et al. 1974). The reaction sequence is initiated by the NADH-dependent incorporation of two hydroxyl groups adjacent to each other on the aromatic ring of the 3-CBA, catalyzed by a very substrate specific benzoic acid 1,2-dioxygenase, capable of dioxygenating benzoic acid and 3-CBA, only (Fig. 2.3). This is a non-regiospecific reaction which results in the dioxygenation at the 1,2- and 1,6-position. Consequently, a mixture of 3- and 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (colloquially also referred to in the literature as 3- and 5-chloro-dihydroxydihydrobenzoic acid), which are then converted to 3- and 4-chlorocatechol, respectively, by a NAD-dependent dehydrogenase. The regiospecificity of B13's benzoic acid 1,2 dioxygenase has also been observed in other benzoic acid utilizing bacteria (Clarke et al. 1975; Horvath and Alexander 1970; Hughes 1965; Spokes and Walker 1974; Walker and Harris 1970; Yamaguchi and Fujisawa 1980; Reber and Thierbach 1980; Zaitsev and

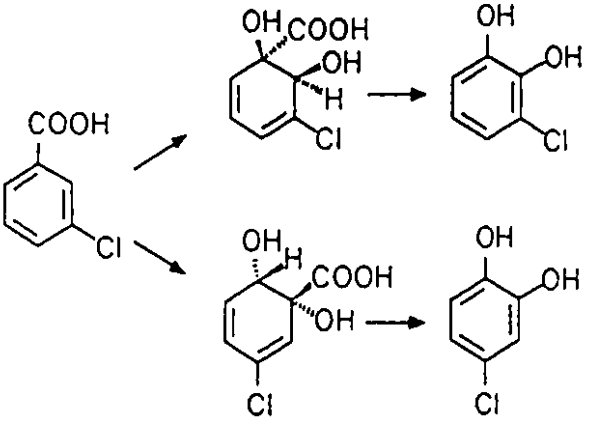


Figure 2.3

The non-selective dioxygenation of 3-chlorobenzoic acid by the benzoic acid 1,2-dioxygenase in *Pseudomonas* sp. B13, forming 3- and 4-chlorocatechol. Adapted from Haggblom (1992).

Baskunov 1985). However, differences in the regiospecificity of this enzyme have been observed in different strains. *Pseudomonas aeruginosa* and two *Azotobacter* strains converted 3-CBA solely into 3-CC (Ichihara et al. 1962; Walker and Harris 1970), whereas an *Arthrobacter* sp. specifically dioxygenated 3-CBA in the 1,6 position yielding 4-CC, which was not metabolized and thus accumulated (Horvath and Alexander 1970).

Dioxygenation of benzoic acid at the 1,2-position by an enzyme with broader substrate specificity than *Pseudomonas* sp. B13's benzoic acid 1,2-dioxygenase, has been reported (Ramos et al. 1987; Williams and Murray 1974; Harayama et al. 1984). This enzyme is in reality a toluic acid dioxygenase encoded by the *meta*-pathway of the TOL plasmid and is capable of accepting 3- , 4-CBA and 3,5-DCBA as substrates (Ramos et al. 1986). However, the chlorinated catechols formed, 4-CC and 3,5-DCC, were not metabolized by the *meta*-cleavage enzyme encoded by the Tol-plasmid (See section 2.2.2.1). A derivative strain, obtained by transferring the Tol-plasmid from *Pseudomonas putida* mt-2 into *Pseudomonas* B13 which possessed the *ortho*-cleavage enzyme capable of dissimilating 4-CC and 3,5-DCC, was able to grow on 3- ,4-CBA and 3,5-DCBA as sole carbon and energy sources (Reineke and Knackmuss 1979). A *Pseudomonas* sp. WR912 isolated by continuous enrichment with 3- , 4-CBA and 3,5-DCBA as sole sources of carbon and energy, degraded these substrates by the same mechanism as the derivative strain of *Pseudomonas* B13 (Hartmann et al. 1979).

2.2.1.1.1 Benzoic acid 1,2-dioxygenases and their evolutionary relatedness

Benzoic acid dioxygenase and the toluic acid dioxygenase belong to a group of enzymes referred to as multicomponent aromatic ring dioxygenases.

As mentioned previously, they catalyze the NADH-dependent incorporation of two adjacent hydroxyl groups on the aromatic ring of benzene, toluene, naphthalene, biphenyl, and benzoic acid and of their chlorinated analogues (Ensley et al. 1983; Furukawa and Miyazaki 1986; Gibson et al. 1990; Harayama et al. 1986; Irie et al. 1987; Mondello 1989; Neidle et al. 1991; Sander et al. 1991; van der Meer et al. 1991a). The oxygen atoms of the two hydroxyl groups are exclusively derived from molecular oxygen (Yamaguchi et al. 1975). Of all the multicomponent aromatic ring dioxygenases, the benzoic acid and the toluic acid dioxygenases have two components instead of three as shown in Fig. 2.4 (Neidle et al. 1991; Moodie et al. 1990; Yamaguchi and Fugisawa 1980).

The hydroxylase component (also referred to as terminal oxygenase, or iron-sulphur-protein) consists of two non-identical protein subunits (α) and (β), with approximate molecular weights of 50 and 20 kDa, respectively. The subunit structure of $\alpha_3\beta_3$ was proposed. An iron-sulphur cluster of the [2Fe-2S] type per $\alpha\beta$ subunit appears to be attached to the α subunit as a prosthetic group. Mononuclear Fe (II) appears to be associated with the α subunit and is believed to bind the oxygen. The N-terminal region of the α-polypeptide may be responsible for the recognition and activation of two oxidizable carbons of the aromatic ring, reception of electrons from the electron transfer component, binding of the β subunit, and the binding and activation of oxygen. The β subunit is believed to be responsible for the enzyme's substrate specificity (Neidle et al. 1991).

The electron transfer component (reductase) is an iron-sulphur flavoprotein (38 kDa), containing one FAD and one iron-sulphur [2Fe-2S] center (Yamaguchi and Fujisawa 1978). The ferredoxin-like structure is in the N-terminal region and an NADH-ferredoxin reductase-like structure is in its C-

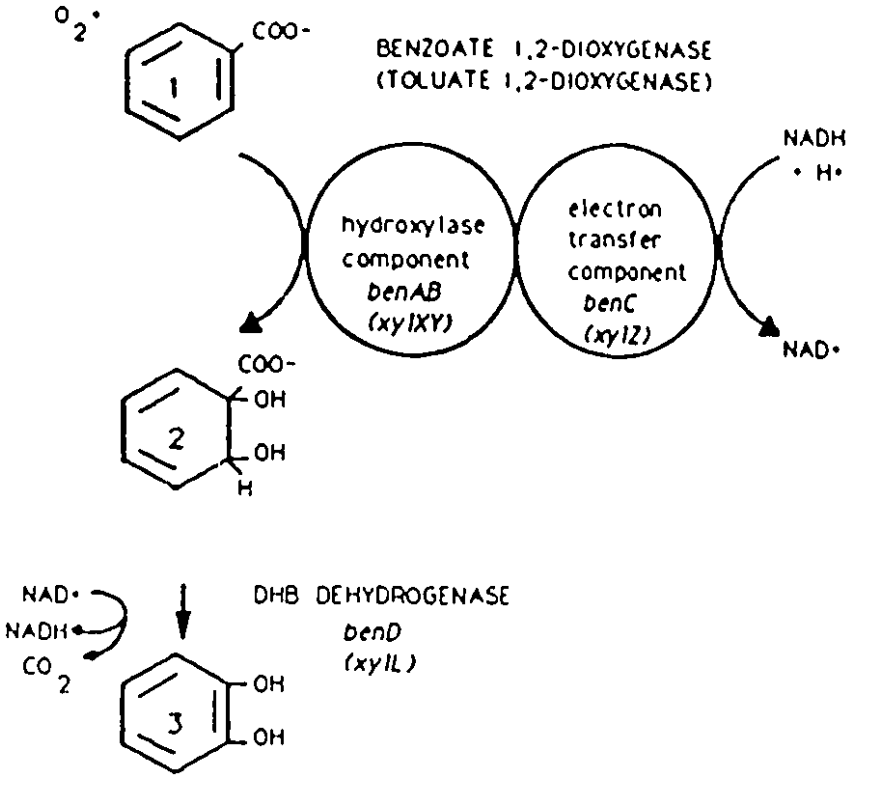


Figure 2.4

Mechanism for the dioxygenation of benzoic acid catalyzed by benzoic acid 1,2-dioxygenase and toluate 1,2-dioxygenase. These two enzymes initiate the conversion of benzoic acid (1) to 2-hydro-1,2-dihydroxybenzoic acid (DHB) (2). The DHB is converted to catechol (3) by an NAD-dependent dehydrogenase. Adapted from Neidle et al. (1991).

terminal region (Neidle et al. 1991). In the three component dioxygenases (naphthalene, toluene and benzene 1,2-dioxygenases), the electron transfer component is composed of two distinct redox proteins. One is a flavoprotein that has NADH oxidase activity, and the other is a ferredoxin which has an [2Fe-2S] cluster (Neidle et al. 1991).

The toluic acid dioxygenase of *P. putida* is encoded by the *xylXYZ* genes of the Tol-plasmid, whereas the benzoic acid 1,2-dioxygenase of *Acinetobacter calcoaceticus* is encoded by chromosomal *benABC* genes. The α and β subunits of the hydroxylase component are encoded by *xylX*, *xylY* and *benA*, *benB* for toluic and benzoic acid dioxygenases, respectively. Deduced amino acid sequence comparisons showed 62% of the aligned BenA and XylX residues were identical, and comparison between XylY and BenB residues revealed 56% homology. A 49% homology of the BenC and XylZ residues, the electron transfer components, was observed. These strong homologies suggest that the genes encoding the benzoic acid and toluic acid 1,2-dioxygenase enzyme complex are derived from common ancestors. Furthermore, comparison of the amino acid sequences of the hydroxylase component among the two- and three-component enzymes, again revealed a common ancestry. In contrast, no apparent evolutionary ancestry was observed among the electron transfer components (Niedle et al. 1991). Indeed, as previously mentioned in the naphthalene, toluene and benzene 1,2-dioxygenases the electron transfer component is composed of two redox proteins, whereas in benzoic acid and toluic acid 1,2-dioxygenases, the transfer of electrons from NADH to the hydroxylase component is achieved by a dual-function protein.

2.2.1.1.2 Dihydrodihydroxybenzoic acid (DHB) and dihydrodihydroxybenzoic dehydrogenase

The non-aromatic DHB (3,5-cyclohexadiene-1,2-diol-1-carboxylic acid or dihydrodihydroxybenzoic acid) is formed as a result of 1,2-dioxygenation of benzoic acid. The re-aromatization of the dihydrodiol derivative to the corresponding catechol is achieved by a NAD+-dependent dehydrogenation, by a dihydrodihydroxybenzoic acid dehydrogenase (Fig. 2.4). Apparently, a free carboxylic group is necessary for substrate binding, since the methyl ester of DHB is inactive as a substrate or inhibitor. The dehydrogenation reaction is believed to form a short-lived intermediate, a β-ketoacid, which decarboxylates spontaneously to catechol (Fig. 2.5). The reactive site is the hydroxyl group at the C-2 (Reineke and Knackmuss 1978b).

Dioxygenation of 3- , 4-CBA, and 3,5-DCBA forms 3- , 5-chlorodihydrodihydroxybenzoic acid and 3,5-dichlorodihydroxybenzoic acid, which are stable compounds. Dehydrogenation of these substituted *cis*-diols forms 3- , 4-CC, and 3,5-DCC, respectively. Comparison of the DHB-dehydrogenase from *A. eutrophus* and *Pseudomonas* sp. B13 revealed very little difference both at the biochemical and kinetic levels (Reineke and Knackmuss 1978b). Halogen or methyl-substituents in the 3- or 5- position has no significant effect on binding of the substrate. However, the enzyme exhibited significantly reduced affinities for 4-substituted DHBs. Inductive rather than steric effects appeared to influence binding affinity, since 4-methyl-DHB was a much better substrate than 4-chloro-DHB.

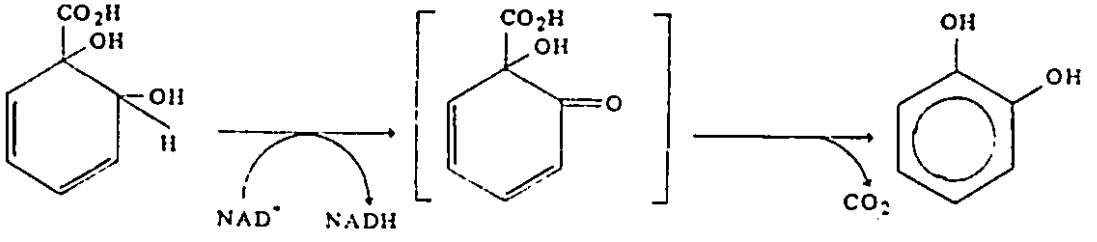


Figure 2.5

Proposed detailed mechanism for the conversion of DHB to catechol via the formation of an unstable β-ketoacid intermediate . Taken from Reineke and Knackmuss (1979).

2.2.1.2 Dehalogenating Pathways

1-Oxygenolytic dehalogenation

2-Hydrolytic dehalogenation

3-Reductive dehalogenation

2.2.1.2.1 Oxygenolytic dehalogenation

The fortuitous dehalogenation by dioxygenases was first reported to occur in the degradation of 2-fluorobenzoic acid (2-FBA) by *Pseudomonas* species (Goldman et al. 1967; Milne et al. 1968). Due to the non-selective dioxygenation by the benzoic acid 1,2-dioxygenase, a mixture of 2- and 6-fluoro-1,2-dihydrodihydroxybenzoic acid was formed. Approximately 85% of the 2-FBA was transformed to catechol, the rest was transformed to 3-fluorocatechol and subsequently to 2-fluoro-*cis*-*cis*-muconic acid (the end product). The nonenzymatic release of the halogen from 2-fluoro-1,2-dihydrodihydroxybenzoic acid was shown from indirect evidence using a mutant, *Alcaligenes eutrophus* defective in the DHB dehydrogenase. This organism utilized 2-FBA as a growth substrate releasing 80% of the originally covalently-bound fluorine (Reiner and Hegeman 1971; Engesser et al. 1980). It was speculated that since fluorine is a leaving group, it is spontaneously eliminated from 2-fluoro-1,2-dihydrodihydroxybenzoic acid as an anion. Apparently, decarboxylation of the resulting β-keto acid forming catechol is likewise non-enzymatic. Therefore, as a result of a non-specific benzoic acid 1,2-dioxygenase, both oxygenolytic dehalogenation and the conversion of halogenated benzoic acid into halocatechol can occur within an organism. This phenomenon does not seem to be exclusive to fluorobenzoic acid-degrading microorganisms. An analogous mechanism has been reported in the degradation of 2-CBA and *ortho*-substituted dichlorobenzoic acids (Zaitsev and

Karasevich 1984; Hartmann et al. 1989; Hickey and Focht 1990; Hernandez et al.1991; Sylvestre et al. 1989). These reports will be discussed separately in section 2.2.1.3. of the literature review.

In contrast to the non-specific dehalogenating dioxygenation of 2-FBA and 2-CBA in the organisms described above, Fetzner and co-workers (1989a, 1989b, 1992) have purified and characterized a very regioselective 2-halobenzoic acid 1,2-dioxygenase from *Pseudomonas cepacia* 2-CBS, which specifically dioxygenates at the 1,2-positions only. Indeed, neither 6-halo-1,2-dihydrodihydroxybenzoic acid nor 3-chlorocatechol, the expected metabolites of a 1,6-dioxygenation, were detected. As with 2-FBA metabolism by *Alcaligenes eutrophus*, it is proposed that the hypothetical 2-halo-1,2-dihydrodihydroxybenzoic acid is highly unstable and during the energetically favourable re-aromatization, CO_2 and halide are spontaneously released. This non-enzymatic decarboxylating-dehalogenating event obviates the need for a functional dehydrogenase (Reiner and Hegeman 1971). The 2-halobenzoic acid 1,2-dioxygenase, is a two-component enzyme system which catalyzes the conversion of 2-fluoro-, 2-chloro-, 2-bromo-, and 2-iodobenzoic acid to catechol in the absence of any detectable dihydrodiol intermediate (Fig. 2.6). In *Pseudomonas cepacia* 2-CBS, the catechol formed is cleaved by the *meta*-cleavage enzyme, catechol 2,3-dioxygenase. This organism utilized 2-CBA, 2-FBA, and 2-bromobenzoic acid as sole carbon sources, but did not use any other mono- or dihalobenzoic acids.

Structurally, benzoic acid 1,2-dioxygenase from *P. putida (arvilla)* C-1, toluic acid dioxygenase from *P. putida (arvilla)* mt-2 and 2-halobenzoic acid 1,2-dioxygenase from P. cepacia 2CBS, are very similar. The reductase (electron transfer) component of the 2-halobenzoic acid 1,2-dioxygenase has a

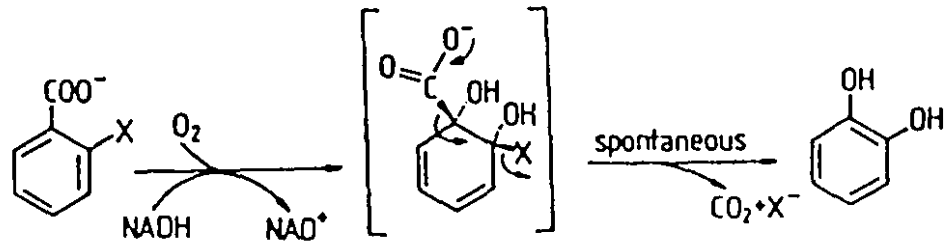


Figure 2.6

Proposed mechanism for the dioxygenation of *ortho*-substituted halobenzoic acids catalyzed by 2-halobenzoic acid 1,2-dioxygenase. **X** can represent F, Cl, Br, I, or NH_2. Taken from Fetzner et al. (1992).

molecular weight of approximately 38 kDa, containing one FAD and a [2Fe-2S] cluster. The hydroxylase component consists of two non-identical protein subunits (α and β) with molecular weights of 52 and 20 kDa, respectively. Similar to benzoic acid 1,2-dioxygenase, the proposed structure of the subunits is $\alpha_3\beta_3$. Three [2Fe-2S] clusters, one per $\alpha\beta$ subunit was suggested (Fetzner et al. 1992). Unlike, benzoic acid 1,2-dioxygenase, mononuclear nonheme Fe(II) was not detected in 2-halobenzoic acid 1,2-dioxygenase, but required exogenous Fe^{2+} for activity. Benzoic acid 1,2-dioxygenase does not require exogenous Fe^{2+} for activity.

Although these dioxygenases are structurally very similar, comparison of the amino acid sequence of the NH_2-terminus between 2-halobenzoic acid 1,2-dioxygenase and toluic acid dioxygenase, revealed no homology (Fetzner et al. 1992).

2.2.1.2.2 Hydrolytic dehalogenation

Hydrolytic dehalogenation reactions involve the displacement of the chlorine from the aromatic ring by a hydroxyl group derived from water rather than molecular oxygen and the reaction can occur in oxygen-free environments (Marks et al. 1984a, 1984b; Muller et al. 1984; van den Tweel et al. 1987). Johnston et al. (1972) first described this type of dehalogenation in the degradation of 3-CBA by *Pseudomonas* sp. The metabolites, 3-hydroxybenzoic acid and 2,5-dihydroxybenzoic acid, were detected in the culture medium of 3-CBA grown cells. It was assumed that 3-CBA degradation proceeded by hydroxylation-dehalogenation forming 3-hydroxybenzoic. This metabolite was then hydroxylated at the 6-position yielding 2,5-dihydroxybenzoic acid.

Initial dehalogenation by hydroxylation of 2-CBA and other *ortho*-substituted halobenzoic acids has been proposed to occur in *Pseudomonas*

aeruginosa (Higson and Focht 1990). The proposed degradation pathway of 2-CBA proceeds by the initial hydrolytic dehalogenation forming 2-hydroxybenzoic acid (salicylic acid) which is then oxidized to catechol.

However, the majority of the reports describing hydrolytic dehalogenation have been in 4-CBA degrading microorganisms, including *Pseudomonas* sp. strain CBS3 (Scholten et al. 1991; Loffler et al. 1991), *Acinetobacter* sp. strain 4-CB1 (Copley and Crooks 1992), *Arthrobacter globiformis* KZT1 (Zaitsev et al. 1991), *Arthrobacter* sp. (Marks et al. 1984a; 1984b), *Alcaligenes denitrificans* NTB-1 (van den Tweel et al. 1987) and *Nocardia* sp. (Klages anf Lingens 1979). Detailed studies of the enzymatic dehalogenation reaction in *Acinetobacter* sp. strain 4-CB1 and *Pseudomonas* sp. strain CBS3 have recently been reported and the mechanisms appear to be virtually identical (Copley and Crooks 1992; Scholten et al. 1991). The dehalogenase enzyme in *Pseudomonas* sp. strain CBS3 is composed of two components, a 4-chlorobenzoate:CoA ligase-dehalogenase (an $\alpha\beta$ dimer of 57 and 30 kDa) and a thioesterase (α_4 tetramer, 65 kDa). In the presence of Mg^{2+}, CoA, H_2O and 4-CBA, the $\alpha\beta$ dimer catalyzes the cleavage of ATP to AMP and PPi coupled with the formation of the 4-HBA:CoA adjunct (Fig. 2.7, reactions 1, 2, and 3). ATP cleavage did not occur in the absence of CoA. The thioesterase catalyzes the hydrolysis of the 4-HBA:CoA thioester to 4-HBA and CoA (Fig. 2.7, reaction 4).

In all cases cited, the subsequent conversion of 4-HBA to 3,4-DHBA is catalyzed by 4-hydroxybenzoic acid-3-hydroxylase, a NAD(P)H-dependent reaction. The fate of the 3,4-DHBA is discussed in section 2.4. *Acinetobacter* sp. strain 4-CB1 hydrolytically dehalogenates 4-CBA and 3,4-DCBA to 4-HBA and 3-chloro-4-hydroxybenzoic acid, respectively. Although, 3,4-DCBA did not induce the initial dehalogenase, 3-chloro-4-hydroxybenzoic acid was utilized as

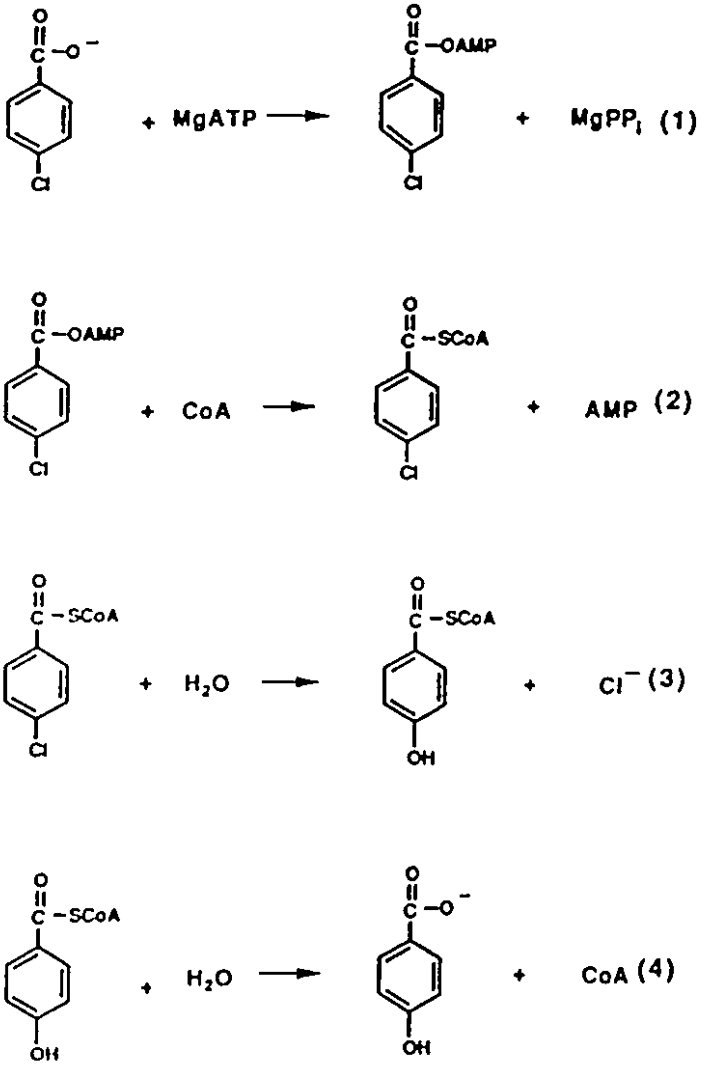


Figure 2.7

Proposed hydrolytic dehalogenation of 4-chlorobenzoic acid. The series of reactions are described in the text. Taken from Scholten et al. (1991).

a sole carbon source by the bacterium. The enzymatic mechanism for the degradation of this latter compound is not known (Adriaens et al. 1991).

2.2.1.2.3. Reductive dehalogenation

The displacement of a halogen, covalently bound to the benzene ring, by a hydrogen atom can occur anaerobically in the presence of an alternate terminal electron acceptor. Several bacterial consortia have been shown to dehalogenate mono- , di- , and trichlorobenzoic acid anaerobically (Mohn and Tiedje 1992). With the exception of the reductive dechlorination of 3-CBA by the Gram-negative, obligately-anaerobic and sulphate reducing, *Desulfomonile tiedjei* strain DCB-1, very little is known about anaerobic dehalogenation of aromatic compounds by pure cultures (De Weerd et al. 1990; Shelton and Tiedje 1984). Reductive dehalogenation by crude lysates of *D. tiedjei* was membrane-associated and dependent on reduced methyl viologen. Dehalogenation was stimulated by formate, carbon monoxide, or H_2 and inhibited by O_2. Thermodynamic data revealed that reductive dechlorination of 3-CBA yielding benzoic acid was an exergonic reaction (Dolfing and Tiedje 1987). This was also observed with a defined 3-CBA degrading methanogenic consortium which resulted in higher cell yields with 3-CBA as a growth substrate than with equimolar concentrations of benzoic acid (Dolfing and Tiedje 1986). Addition of 3-CBA to starved cells of DCB-1 coincided with an increase in the ATP-levels in the cells. Furthermore, oxidation of formate could be coupled to the reductive dechlorination of 3-CBA or 3,5-DCBA, and growth yields increased proportionately with levels of dechlorination. However, formate and H_2 did not support substrate-level phosphorylation. Hence, energy conservation from the metabolism of formate or H_2 plus 3-CBA must be respiratory. Mohn and Tiedje (1991) have recently suggested a chemiosmotic

coupling of dechlorination and ATP synthesis (Fig. 2.8). Formate dehydrogenase and the putative dechlorinating enzyme may be vectorially located resulting in the extrusion of protons from the cell. In the presence of a membrane bound ATPase, the resulting proton motive force supports ATP synthesis. However, this is still speculative since the actual mechanism of the dehalogenation is still not known and the isolation and purification of the dechlorinating enzymes have not been performed (Mohn and Tiedje 1992).

Recently, dehalogenation of 2,4-DCBA under aerobic conditions has been proposed to occur in a coryneform bacterium NTB-1 (van den Tweel et al. 1987). Reductive dehalogenation of 2,4-DCBA forming 4-CBA was suggested on the basis of the presence or absence of key intermediates. The chlorinated intermediate, 4-CBA was then hydrolytically dechlorinated to 4-HBA as described in section 2.2.1.2.2. NTB-1 cells did not utilize 2-chloro-4-hydroxybenzoic acid, nor was this compound identified as a metabolite, indicating that removal of the chlorine at the C-2 position occured prior to the hydrolytic dehalogenation event. This was further illustrated by the fact that when 4-CBA and 2,4-DCBA were added simultaneously to resting cells, the concentration of 4-CBA remained constant until most of the 2,4-DCBA was depleted. The 2,4-DCBA was not degraded by the non-dehalogenating pathway (section 2.2.1.1), since 3,5-DCC the theoretical intermediate of such a route, was not metabolized by NTB-1. These observations led the authors to conclude that in the coryneform bacterium NTB-1 (previously identified *Alcaligenes denitrificans*), 2,4-DCBA was reductively dehalogenated to 4-CBA and hydrolytically dehalogenated 4-CBA to 4-HBA. Attempts to demonstrate the presence of both the reductive 2,4-DCBA and the hydrolytic 4-CBA dehalogenases were unsuccessful (van den Tweel et al. 1987).

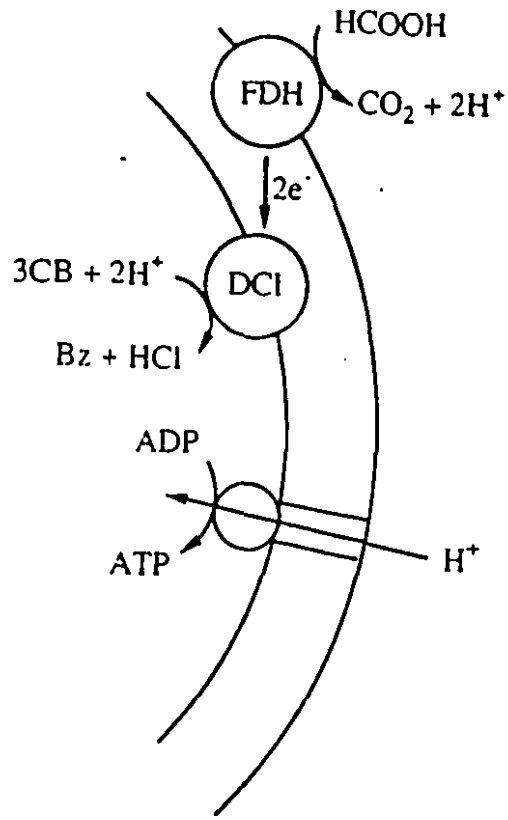


Figure 2.8

Chemiosmotic coupling model for the reductive dehalogenation of 3-chlorobenzoic acid (3-CB) to benzoic acid (Bz). The proton motive force couples reductive dehalogenation and ATP synthesis by a proton-driven ATPase. Abbreviations: DCl, putative dechlorinating enzyme; FDH, formate dehydrogenase. Taken from Mohn and Tiedje (1992).

2.2.1.3 Variations on a Theme

The degradation of 2-FBA, mainly through oxygenolytic dehalogenation by *Alcaligenes eutrophus* and *Pseudomonas* sp., was described in section 2.2.1.2.1. However, due to the non-selective dioxygenation event, a fluorocatechol was also formed, analogous to the non-dehalogenating pathways described in section 2.2.1.1. This section will describe several microorganisms capable of degrading *ortho*-substituted mono- and dichlorobenzoic acids, via oxygenolytic dehalogenation as described in section 2.2.1.2.1. and via dioxygenation without dehalogenation as described in section 2.2.1.1. The hybrid *Pseudomonas* sp. JH230, capable of growth on 2-CBA was constructed *in vivo* by mating *Pseudomonas* sp. WR401 and *Pseudomonas* sp. B13. Strain WR401 enriched on methylsalicylates (methyl-substituted 2-hydroxybenzoic acid) possessed a non-specific benzoic acid 1,2-dioxygenase and the *meta*-cleavage enzyme, catechol 2,3-dioxygenase, which is not desirable for the cleavage of chlorinated catechols (see section 2.2.2.1). *Pseudomonas* sp. B13 possessed a very specific benzoic acid 1,2-dioxygenase (high specificity for B.A. and 3-CBA) and a broad substrate *ortho*-cleavage enzyme, chlorocatechol 1,2-dioxygenase, capable of cleaving chlorocatechols forming non-toxic intermediates. The hybrid strain possessed the broad substrate(non-selective) benzoic acid 1,2-dioxygenase and the chlorocatechol 1,2-dioxygenase. The degradation of 2-CBA by the hybrid strain proceeded via the non-selective 1,6-dioxygenation forming a stable 6-chloro-1,2-dihydroxydihydrobenzoic acid which was subsequently dehydrogenated to 3-CC. The latter metabolite was cleaved by chlorocatechol 1,2-dioxygenase which was induced by 2-CBA (Hartmann et al. 1989). The broad substrate specificity of the benzoic acid 1,2-dioxygenase allowed the organism to also transform benzoic acid, 3- , 4-CBA, 2,4- , and 3,5-DCBA.

A natural isolate, *Pseudomonas aeruginosa* JB2, enriched on 2-CBA, also degraded the substrate solely via 1,6-dioxygenation forming 6-chloro-1,2-dihydroxydihydrobenzoic acid and 3-CC. Whereas, 3-CBA was specifically degraded via 1,2-dioxygenation forming 3-chloro-1,2-dihydroxydihydrobenzoic acid and 3-CC (Hickey and Focht 1990).

Pseudomonas sp. strain B-300, a natural isolate, degraded 2-CBA via catechol to *cis-cis*-muconic, and via 3-CC to 2-chloro-*cis-cis*-muconic acid (Sylvestre et al. 1989). This again implies a non-selective dioxygenation in the 1,2 and 1,6 positions. The presence of a chlorocatechol 1,2-dioxygenase was assumed but not shown. The mechanism of 2-CBA dioxygenation appears to be homologous to the 1,2 and 1,6-dioxygenation of 2-FBA by *Alcaligenes eutrophus* (Reiner and Hegeman 1971) and *Pseudomonas* sp. (Goldman et al. 1967). It is possible that *Pseudomonas* sp. strain B-300 possesses a benzoic acid dioxygenase which dioxygenates at the 1,6-position forming 3-CC, and a 2-chlorobenzoic acid 1,2-dioxygenase which specifically dioxygenates at the 1,2-position thus forming catechol (Dr. M Sylvestre, personal communication).

An interesting variation of the long held views of *ortho*-substituted mono- and dichlorobenzoic acid degradation was presented by Hickey and Focht (1990) and Hernandez et al. (1991). *Pseudomonas putida* strain P111 degrades 2- , 3- , 4-CBA, 2,3- , 2,4- , 2,5- , 3,5-DCBA and 2,3,5-TCBA. The degradation of 4-CBA is proposed to proceed via 1,2-dioxygenation yielding a stable intermediate, 4-chloro-DHB, which is subsequently dehydrogenated to 4-CC, analogous to the scheme postulated by Hartmann et al. (1979) and described in section 2.2.1.1. This belief was reinforced by the failure to identify 4-HBA in the growth medium and by the lack of 4-CBA transformation under a reduced oxygen concentration thereby not implicating hydrolytic dehalogenation. Growth of strain P111 on all the *ortho*-substituted

chlorobenzoic acids did not induce dihydrodiol (DHB) dehydrogenase activity. This suggests that specific 1,2-dioxygenation of the *ortho*-substituted benzoic acids occured forming unstable 2-chloro- , 2,3-dichloro- , 2,4-dichloro- , 2,5-dichloro- , and 2,3,5-trichloro-1,2-dihydrohydroxybenzoic acid from 2-CBA, 2,3-, 2,4- , 2,5-DCBA, and 2,3,5-TCBA, respectively (Fig. 2.9). These unstable chlorinated dihydrodiols spontaneously decarboxylate and lose the *ortho*-chlorine forming the corresponding catechol or chlorinated catechol. Although the postulated benzoic acid 1,2-dioxygenase has not been isolated, its activity in whole cell assays resembles that of the 2-halobenzoic acid 1,2-dioxygenase from *P. cepacia* 2CBS (Fetzner et al. 1992) but has a much broader substrate specificity. Induction of a dihydrodiol dehydrogenase in strain P111 was achieved by growth on 3- and 4-CBA. Dioxygenation of 3- , and 4-CBA at the 1,2 positions yields stable chlorinated dihydrodiols, necessitating a functional dihydrodiol dehydrogenase (Fig. 2.9). Interestingly, strain P111 does not utilize 3,5-DCBA as a growth substrate. However, resting cells grown on 3- , or 4-CBA readily metabolized this compound. Dioxygenation at the 1,2 positions of the 3,5-DCBA yields a stable dihydrodiol intermediate, but this substrate does not induce the synthesis of the DHB dehydrogenase. A similar mechanism of the dioxygenation of 2,5-DCBA was described for *Pseudomonas aeruginosa* JB2 (Hickey and Focht 1990).

2.2.2 Dissimilation of catechol and chlorocatechols

In previous sections, the different means by which microorganisms convert BP, CBP, BA and CBAs to the catecholic or chlorocatecholic intermediates have been discussed. An interesting variation exists in the mechanism by which these dihydroxylated (catecholic) intermediates are de-aromatized. The catecholic intermediates are channelled into one of two

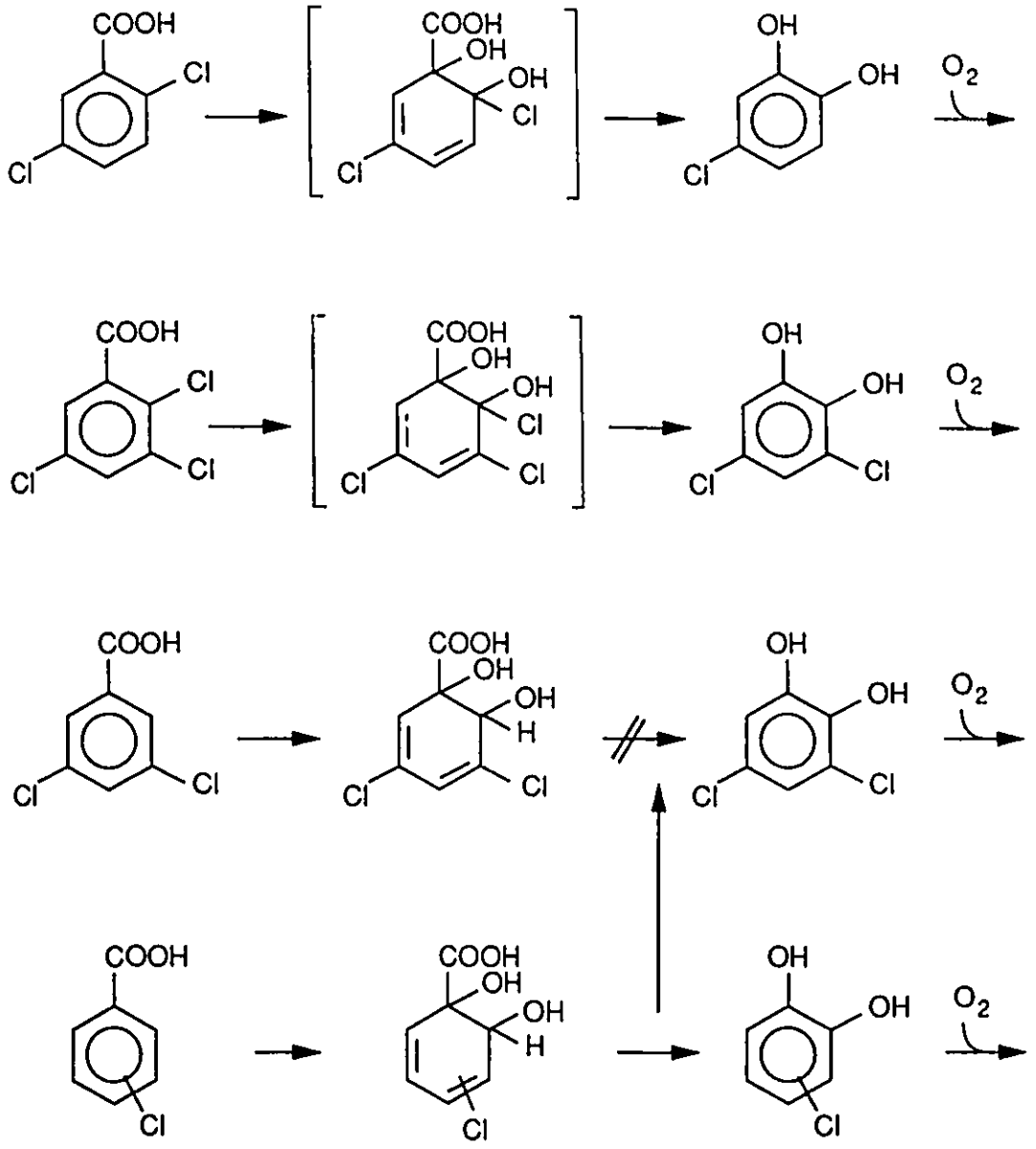


Figure 2.9

Proposed metabolism of chlorobenzoic acids by *P. putida* P111. Bracketed compounds are unstable and spontaneously decarboxylate and lose *ortho*-chlorines. Growth on *ortho*-substituted chlorobenzoic acids does not induce a functional dihydrodiol dehydrogenase, whereas growth on 3- or 4-chlorobenzoic acid does. Taken from Hernandez et a. (1991).

possible pathways, either a *meta*- or an *ortho*-cleavage type pathway. The choice is microorganism-dependent, and both pathways lead to intermediates of the central metabolic pathways such as the tricarboxylic acid cycle. Both pathways are initiated by distinct and specialized enzymes which incorporate molecular oxygen into catecholic intermediates.

2.2.2.1 *Meta*-cleaving enzymes and *meta*-cleavage of chlorocatechol

Meta-cleavage enzymes are non-heme iron dioxygenases which contain Fe(II) as a cofactor (Wallis and Chapman 1990). They cleave the aromatic ring adjacent to the hydroxyl groups and are also referred to as extradiol dioxygenases (Fig. 2.10). These enzymes include the catechol 2,3-dioxygenases encoded by *xylE* gene of the Tol-plasmid (Wallis and Chapman 1990), and *nahH* gene of the Nah-plasmid (Harayama and Rekik 1989); the 2,3-dihydroxy-naphthalene dioxygenase encoded by the *nahC* gene of the NAH-plasmid (Harayama and Rekik 1989), the 2,3-dihydroxybiphenyl 1,2-dioxygenases encoded by the *bphC* gene of the bph-operon (Furukawa et al. 1987; Taira et al. 1988) and the 3-methylcatechol 2,3-dioxygenase encoded by the *todE* of the Tod-operon (Zylstra and Gibson 1989). These enzymes share nucleotide and amino acid sequence similarities and are believed to be closely related (van der Meer et al. 1992). However, the catechol 2,3-dioxygenase from *Alcaligenes eutrophus* JMP222 has little homology at the nucleotide sequence level and is believed not to be related to the other extradiol dioxygenases (Kabisch and Fortnagel 1990).

Meta-cleavage of chlorinated catechols is generally considered to be unproductive. Growth on a chlorinated aromatic compound yielding a chlorinated catechol usually results in the induction of an *ortho*-cleavage

Meta

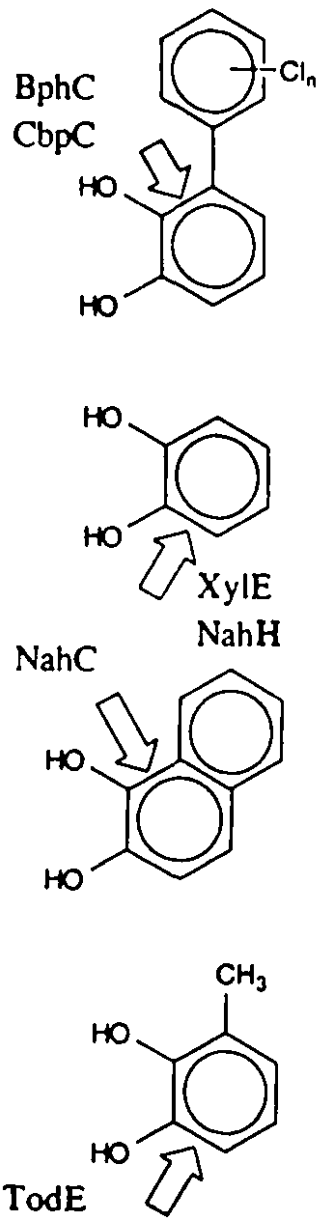


Figure 2.10

Meta-cleaving dioxygenases and their genetic origin. Catechol 2,3-dioxygenase, XylE, NahH ; 2,3-dihydroxybiphenyl 1,2-dioxygenase, BphC, CbpC ; 2,3-dihydroxy-naphthalene 1,2-dioxygenase, NahC ; 3-methylcatechol 2,3-dioxygenase, TodE. Open arrows indicate the site of the ring cleavage. Adapted from van der Meer et al. (1992).

enzyme. *Meta*-cleavage of 3-CC results in an acylhalide which acts as an acylating agent and inactivates the *meta*-cleaving enzyme irreversibly, resulting in the lethal accumulation of catechols (Knackmuss 1981; Furukawa and Chakrabarty 1982; Pettigrew et al. 1990; Reineke et al. 1982; Klecka and Gibson 1981; Bartels et al. 1984). *Meta*-cleavage of other chlorocatechols yields halo-2-hydroxymuconic semialdehydes which are generally toxic to the organism. For example, 5-chloro-2-hydroxymuconic semialdehyde which results from the *meta*-cleavage of 4-CC inhibits catechol 2,3-dioxygenase activity (Commandeur and Parsons 1990; Klecka and Gibson 1981). Productive *meta*-cleavage of 4-CC has been reported in *Pseudomonas cepacia* MB2. The addition of 4-CC to the growth medium did not inhibit growth on 2-methylbenzoic acid. However, catechols bearing a chlorine substituent adjacent to a hydroxyl (3-CC, 3,4- , 3,5- , and 3,6-DCC) were potent inhibitors (Higson and Focht 1992). Productive *meta*-cleavage of 4-CC was also reported in *Pseudomonas putida* mt-2 (Nozaki et al. 1970) and *Alcaligenes faecalis* (Surovtseva et al. 1981). Several cases have been reported where both *meta*- and *ortho*-cleavage enzymes have been induced within the same organism. A *Pseudomonas* sp. possessed both enzymes but growth on CBAs occurred only upon inactivation of the *meta*-cleavage enzyme (Reineke et al. 1982). *Pseudomonas* sp. strain JS6 simultaneously degraded toluene (which induces catechol 2,3-dioxygenase) and chlorobenzene (which induces catechol 1,2-dioxygenase), although better growth yields were obtained when grown separately on either carbon source (Pettigrew et al. 1991). It was proposed that transient accumulation of 3-chlorocatechol, inactivated catechol 2,3-dioxygense activity. *Pseudomonas* sp. strain JS150, grown on chlorobenzene induced both catechol 2,3-dioxygenase and chlorocatechol 1,2-dioxygenase activities (Haigler et al. 1992). A recombinant *Pseudomonas* sp.

CB15 grown on 3-CBP induced the *meta*-cleavage enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase and a chlorocatechol 1,2-dioxygenase (Adams et al. 1992). These examples demonstrate that the simultaneous induction of both the *meta*- and *ortho*-cleavage enzymes is not entirely incompatible.

The *Pseudomonas cepacia* strain MB2, previously mentioned, is unique in its ability to degrade a benzoic acid molecule which bears both chlorine and methyl substituents (3-chloro-2-methylbenzoic acid) via the *meta*-cleavage pathway. Dioxygenation of this substituted benzoic acid yields 4-chloro-3-methylcatechol which upon *meta*-cleavage is proposed to form an aliphatic hydrocarbon, 2-hydroxy-5-chloro-6-oxo-hepta-2,4-dienoic acid which is further metabolized to acetate, pyruvate and chloroacetaldehyde (Higson and Focht 1992).

2.2.2.2 *Ortho*-cleavage pathway of catechol, protocatechuic acid and chlorocatechols

Ortho-cleavage pathways are involved in the degradation of catechol, chlorinated catechols and protocatechuic acid. The degradation of catechol and protocatechuic acid via the *ortho*-degradation pathway yields a common intermediate compound, 3-ketoadipate enol-lactone (also known as 3-oxoadipate enol-lactone) (Fig. 2.11). This compound is then hydroxylated forming 3-ketoadipic acid (β-ketoadipic acid), which picks up CoA from succinyl-CoA to form the intermediate 3-ketoadipyl-CoA. Cleavage of 3-ketoadipyl-CoA yields acetyl-CoA and succinic acid which can then enter the cell's tricarboxylic acid cycle. The series of reactions leading to the formation of the 3-ketoadipic acid from catechol and protocatechuic acid are analogous, with the exception of the decarboxylation event in the protocatechuic acid

degradation pathway (Fig. 2.11). The genes encoding *ortho*-cleavage pathways of catechol and protocatechuic acid are clustered in operons and usually located on the chromosome (Doten et al.1987; Hughes et al. 1988; Neidle and Ornston 1986; Aldrich et al. 1987). A plasmid-encoded catechol 1,2-dioxygenase from a phenol-degrading *Pseudomonas* sp. strain EST1001, has recently been reported (Kivisaar et al. 1991). The genes encoding the enzymes of the chlorocatechol pathway are located on plasmids (Chatterjee et al. 1981; Ghosal and You 1989). Although the reactions involved in the degradation of chlorocatechols are analogous to those involved in the degradation of catechol, significant differences are note-worthy and will be discussed in the following paragraphs.

The degradation of 3- , and 4-CC results in the formation of maleylacetic acid (Dorn et al. 1974; Schmidt and Knackmuss 1980; Dorn and Knackmuss 1978a, 1978b; Frantz and Chakrabarty 1987) (Fig. 2.12). A chromosomally encoded maleylacetic acid reductase is thought to convert maleylacetic acid to β-ketoadipic acid. The isomerization to the enol-lactone by the muconolactone isomerase in the degradation of catechol (Fig. 2.11), is nonenzymatic in the chlorocatechol degradation pathway. Schmidt and Knackmuss (1980) proposed 4-carboxychloromethylbut-2-en-4-olide as an intermediate in the cycloisomerization of 2-chloro-*cis*-*cis*-muconic acid in enzyme preparations of *Pseudomonas* sp. B13 grown on 3-CBA. However, 4-carboxychloromethylbut-2-en-4-olide is unstable and spontaneously generates *trans*-4-carboxymethylenebut-2-en-4-olide by *anti* elimination of hydrogen chloride. Similarly, *cis*-4-carboxymethylenebut-2-en-4-olide is formed from the enzymatic cycloisomerization of 3-chloro-*cis*-*cis*-muconic acid. The 4-carboxymethylenebut-2-en-4-olides are converted to maleylacetic acid by a

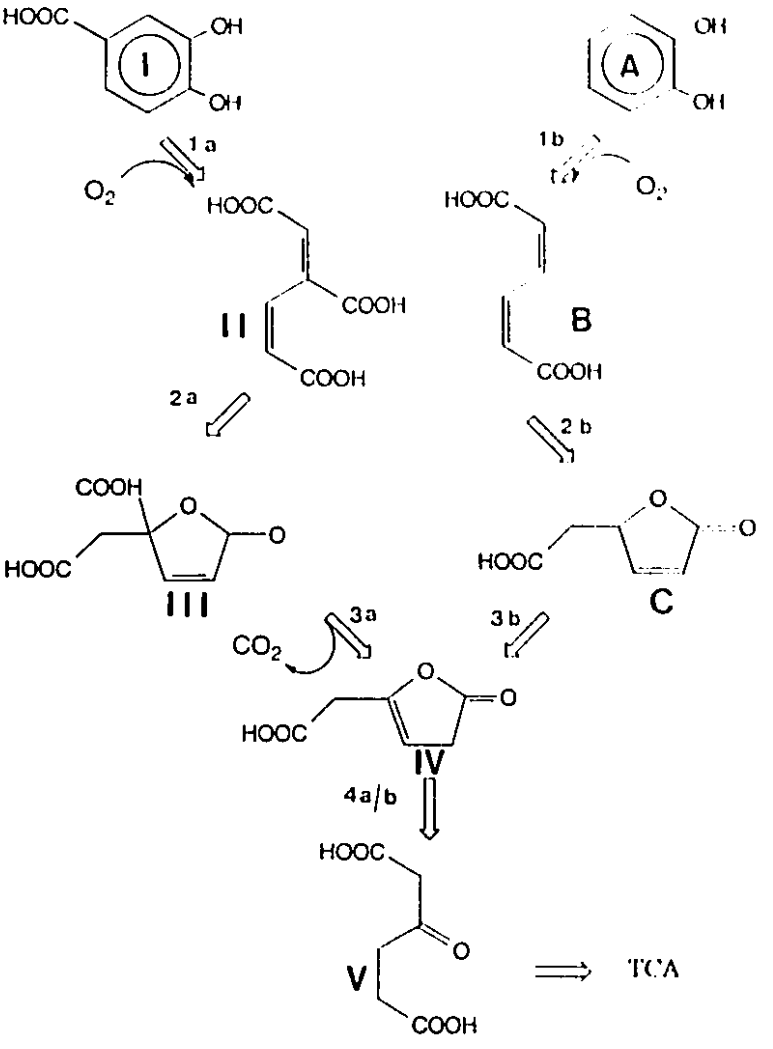


Figure 2.11

Ortho-cleavage pathways for the degradation of protocatechuic acid and catechol. Enzymatic steps for the degradation of protocatechuic acid: (1a) protocatechuic acid 3,4-dioxygenase ; (2a) muconate cycloisomerase; (3a) decarboxylase; (4a) enol-lactone hydrolase. Enzymatic steps for the degradation of catechol: (1b) catechol 1,2-dioxygenase; (2b) lactonizing enzyme; (3b) muconolactone isomerase, (4b) enol-lactone hydrolase. TCA, tricarboxylic acid cycle. *Compounds*: (I) protocatechuic acid; (II) 3-carboxy-*cis-cis*-muconic acid; (III) 4-carboxymuconolactone; (IV) 3-ketoadipate enol-lactone; (V) 3-ketoadipic acid; (A) catechol; (B) *cis-cis*-muconic acid; (C) muconolactone. Adapted from van der Meer et al (1992).

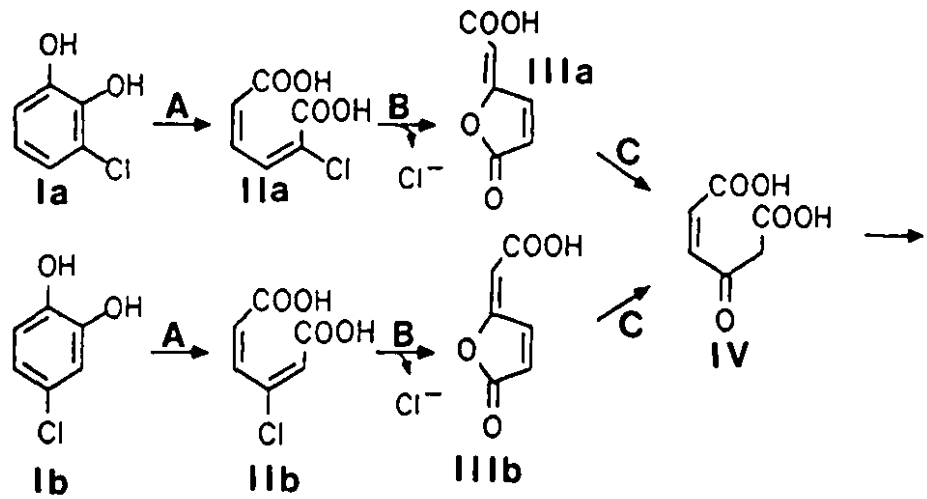


Figure 2.12

Ortho-cleavage pathways for the degradation of 3- and 4-chlorocatechol. *Enzymes* : (A) chlorocatechol 1,2-dioxygenase; (B) chloromuconic acid cycloisomerase; (C) 4-carboxymethylene-but-2-en-4-olide hydrolase.

Compounds : (Ia) 3-chlorocatechol; (IIa) 2-chloro-*cis-cis*-muconic acid; (IIIa) *trans*-4-carboxymethylenebut-2-en-4-olide; (IV) maleylacetic acid; (Ib) 4-chlorocatechol; (IIb) 3-chloro-*cis-cis*-muconic acid; (IIIb) *cis*-4-carboxymethylenebut-2-en-4-olide. Adapted from Haggblom (1992).

hydrolase and further degraded to β-ketoadipic acid (Reineke and Knackmuss 1988).

Although the 3-CBA degrading *Pseudomonas* sp. strain B13 and the 2,4-dichlorophenoxyacetic acid (2,4-D) degrading *A. eutrophus* JMP134 are capable of degrading 3,5-DCC, only recently has the complete degradation pathway of this latter compound been elucidated (Schwien et al.1988; Pieper et al. 1991) (Fig. 2.13). The illusive intermediate of the pathway had been 2,4-dichloro-*cis*-*cis*-muconic acid, which is chemically unstable (Pieper et al. 1991). Like the degradation of 3- and 4-CC, spontaneous dechlorination occurs as a result of the cycloisomerization of the dichloromuconic acid. Two different pathways have been suggested for the further metabolism of 2-chloromaleylacetic acid. One involves the reduction of the double bond via a NADH-dependent reaction forming 5-chloro-3-ketoadipic acid which is subsequently cleaved to acetyl-CoA and chlorosuccinic acid. The other suggested mechanism is the reduction of 2-chloromaleylacetic acid to 3-ketoadipic acid using two moles of NADH, which is then cleaved to form acetyl-CoA and succinic acid (Schwien et al. 1988). The degradation of 3,4-DCC by the dichlorobenzene degrading *Pseudomonas* sp. strain P51 follows the same sequence of reactions as shown in Fig 2.13, forming 5-chloromaleylacetic acid (van der Meer et al. 1991b). The enzymes, chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase are relatively non-specific having high catalytic activity for chlorinated substrates. Chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase retain diminished activity towards non-chlorinated substrates. However, dienelactone hydrolase is highly specific for dienelactones and has no activity toward enol-lactones (Frantz and Chakrabarty 1987).

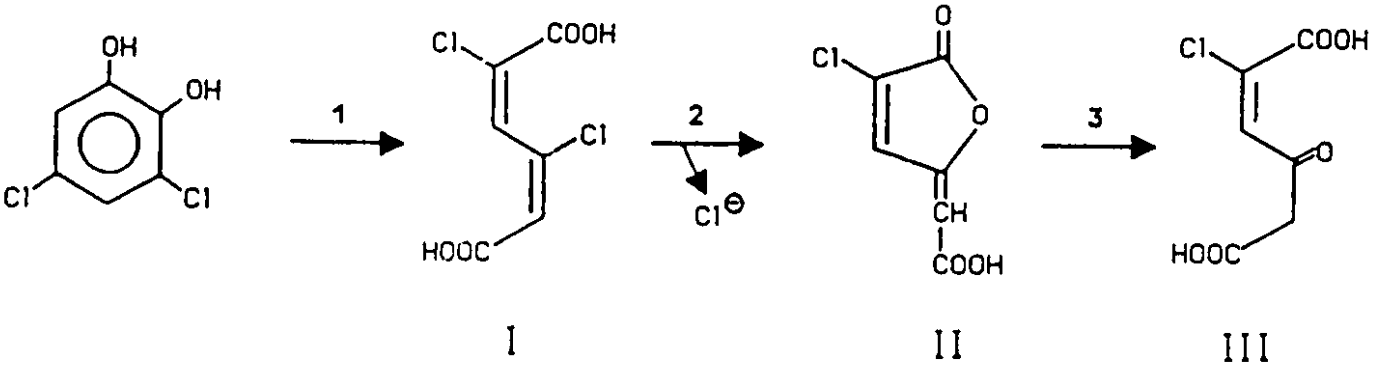


Figure 2.13

Ortho-cleavage pathway for the degradation of 3,5-dichlorocatechol. *Compounds*: (I) 2,4-dichloro-cis-cis-muconic acid; (II) 2-chloro-4-carboxymethylenebut-2-en-4-olide; (III) 2-chloromaleylacetic acid. *Enzymes*: (1) chlorocatechol 1,2-dioxygenase; (2) dichloromuconic acid cycloisomerase; (3) dienelactone hydrolase. Taken from Pieper et al. (1991).

2.2.2.3 *Ortho*-cleaving (intradiol) enzymes

Ortho-cleaving enzymes incorporate both atoms of molecular oxygen into catechol as they cleave between its hydroxylated carbons forming *cis, cis*-muconic acid. Like the *meta*-cleaving enzymes, *ortho*-cleaving enzymes contain non-heme iron. However, unlike the *meta*-cleaving enzymes, the iron cofactor in the *ortho*-cleaving enzymes is in the Fe(III) state (Wallis and Chapman 1990; Broderick and O'Halloran 1991).

The oligomeric structure and the number of ligated ferric irons vary substantially within the intradiol dioxygenases. The catechol 1,2-dioxygenase encoded by the *catA* gene in *Acinetobacter calcoaceticus*, is formed by association of identical protein subunits, each of which contains a ferric ion [2 irons/$\alpha\alpha$ homodimer] (Patel et al. 1976). *Pseudomonas arvilla* C-1, in contrast, has three isoenzymes of catechol 1,2-dioxygenase with equal catalytic capabilities, differing in oligomeric structure, $\alpha\alpha$ (60,000 Da), $\alpha\beta$ (62,000 Da), and $\beta\beta$ (64,000 Da). Each holoenzyme contains only one ferric ion (Nakai et al. 1990).

Protocatechuic acid 3,4-dioxygenases, in general, are formed by association of nonidentical protein subunits with one ferric ion per protomer (Yoshida et al. 1976).

The chlorocatechol 1,2-dioxygenase encoded by the *clcA* gene of pAC27 is a homodimer (57.5 kDa) containing only one ferric ion [α_2Fe] (Broderick and O'Halloran 1991). The structure of the active site of the subunits not containing the iron is not known. The active site of the β-subunit of the protocatechuic acid 3,4-dioxygenase, which contains the Fe (III) has been characterized. The active site is iron coordinated by two tyrosines, two histidines, and one hydroxide (Ohlendorf et al. 1988). It is believed that the catecholic substrates interact directly with the ferric ion via their hydroxyl groups (True et al. 1990). Based on

the nucleotide sequence analysis of the genes encoding intradiol dioxygenases, it appears that the active site in all these enzymes is remarkably conserved and believed to be virtually identical (Hartnett et al. 1990; Kivisaar et al. 1991; Neidle et al. 1988; Perkins et al. 1990; van der Meer et al. 1991b).

Although chlorocatechol 1,2-dioxygenases have a wide substrate range, capable of cleaving catechol, 3- , 4-CC, 3,4- , 3,5- , 3,6-DCC and 3,4,6-TCC, differences in substrate affinities and conversion rates have been observed. These differences have been observed in different microorganisms and appear to be related to the chlorinated aromatic substrate capable of being metabolized by the microorganism and to the chlorinated catechol intermediate formed as a consequence of such metabolism.

The preferred chlorinated aromatic substrate for *Pseudomonas* sp. P51 is 1,2-dichlorobenzene. The degradation of this substrate results in the formation of 3,4-DCC which is efficiently cleaved by the chlorocatechol 1,2-dioxygenase (TcbC) found in the organism. The conversion rates of 3,6-DCC and 3,4,6-TCC are significantly lower. The latter chlorinated catechols are the expected intermediates of 1,4-di- , and 1,2,5-trichlorobenzene, which are poor growth substrates. Interestingly, this organism does not grow on 1,3-dichlorobenzene, although 3,5-DCC, which is the expected intermediate, can be a substrate for the enzyme. The substrate range limitation is therefore upstream of the intradiol cleavage (van der Meer et al. 1991b).

In *Alcaligenes eutrophus* JMP 134, the degradation of 2,4-D results in the formation of 3,5-DCC, which is the preferred substrate of the chlorocatechol 1,2-dioxygenase (tfdC) found in the organism. This enzyme also has high relative activity for 3-CC which is the catecholic intermediate of 3-CBA degradation by strain JMP 134 (Pieper et al. 1988).

The chlorocatechol 1,2-dioxygenases (ClcA) obtained from *Pseudomonas* sp. B13 and *P. putida* show the highest rate of conversion with 3-CC. This is the intermediate expected from the degradation of 3-CBA, which is the only chlorinated benzoic acid that can serve as a growth substrate (Broderick and O'Halloran 1991).

2.2.2.4 Genes encoding enzymes of the chlorocatechol degradation pathway

Genes encoding the enzymes of the chlorocatechol degradation pathway are located on catabolic plasmids. These genes and the plasmids on which they are found have been extensively characterized in different bacteria. *Pseudomonas* sp. B13 carries a plasmid designated pB13 (or pWR1). A *Pseudomonas putida*, which also degrades 3-CBA via the chlorocatechol degradative pathway, carries the pAC25 plasmid. Both plasmids are conjugative and homologous both by DNA-DNA hybridization and by restriction endonuclease digestion profiles (Chatterjee et al. 1981; Chatterjee and Chakrabarty 1983). However, pAC25 is 6-kb larger than pWR1. This additional DNA fragment is not required for 3-CBA degradation or transfer of the plasmid. Indeed, complete degradation of 3-CBA was achieved with a 6-kb deletion derivative of pAC25, designated pAC27 (Ghosal et al. 1985). Subsequently, all genetic studies have proceeded with plasmid pAC27. A complete nucleotide sequence determination of the genes encoding the enzymes for the degradation of chlorocatechols, revealed that three critical genes were clustered within a 4.3-kb DNA fragment subsequently referred to as the *clc ABD* operon. Genes *clcA*, *clcB*, and *clcD* encode for chlorocatechol 1,2-dioxygenase (33 kDa), chloromuconic acid cycloisomerase (40 kDa), and dienelactone hydrolase (28 kDa), respectively (Ghosal and You 1989). Genes *clcB* and *clcD*

are separated by an open reading frame which does not appear to be translated. Immediately preceding the 4.3-kb fragment, there is a 385-bp fragment containing the operator/promoter sequences (Frantz and Chakrabarty 1987).

Alcaligenes eutrophus strain JMP 134, capable of growth on 2,4-D and 3-CBA, harbours the catabolic plasmid pJP4. This plasmid contains the operon *tfd CDEF*, encoding for the chlorocatechol degrading enzymes. Genes *tfdC*, *tfdD* and *tfdE* encode for the analogous enzymes encoded by *clcA*, *clcB*, *clcD*, respectively (Ghosal and You 1989). Gene *tfdF* had been assumed to encode a chloro-*trans*-dienelactone isomerase (Don et al.1985). However, this enzyme is not required for the degradation of 3,5-DCC by *A. eutrophus* JMP 134 and *Pseudomonas* sp. B13. Consequently , the function of *tfdF* gene is presently not known (Pieper et al. 1991). Shlomann et al. (1990) suggested that this gene may encode 5-chloro-3-oxoadipate-dehalogenating activity. In contrast to pAC27, plasmid pJP4 also carries genes *tfdA* and *tfdB* encoding 2,4-dichlorophenoxyacetic acid monooxygenase and dichlorophenol hydroxylase, respectively (van der Meer et al. 1992). Also, genes *tfdD* and *tfdE* are contiguous in the *tfdCDEF* operon, whereas the corresponding genes, *clcB* and *clcD*, of the *clcABD* operon are separated by an open reading frame (Ghosal and You 1989).

Recently, the catabolic plasmid pP51 (110 kb), harboured by the di- and tri-chlorinated benzene-degrading *Pseudomonas* sp. P51, has been characterized (van der Meer et al. 1991a). Plasmid pP51 contains two gene clusters that encode the entire degradation of chlorinated benzenes, *tcbAB* which encodes the enzymes for the conversion of chlorinated benzenes to chlorinated catechols (benzene dioxygenase and a dehydrogenase, respectively) and *tcbCDEF* (5.5 kb) which encodes the enzymes of the

chlorocatechol degradative pathway. The *tcbCDEF* gene cluster shows strong DNA homology (between 57.6 and 72.1%, identity) and an organization similar to the *clcABD* and *tfdCDEF* operons. The *tcbCDEF* and *clcABD* gene clusters are more closely related to each other than to *tfdCDEF* (van der Meer et al. 1991b). The *tcb* cluster possesses an extra reading frame, designated *tcbF* which is homologous to the *tfdF* gene. As is the case with *tfdF*, the *tcbF* gene product is not known. Similar to the *clcABD* operon, the *tcb* cluster is not contiguous, having an open reading frame separating *tcbD* and *tcbE*. Genes *tcbC*, *tcbD* and *tcbE* encode enzymes analogous to those encoded by *clcA*, *clcB*, and *clcD*, respectively.

Based on the degree of identity of deduced amino acid (a.a.) sequences, it has been suggested that intradiol-cleaving enzymes are evolutionarily related. However, the chlorocatechol 1,2-dioxygenases appear to have evolved forming a separate group. The a.a. sequence homology amongst the chlorocatechol 1,2-dioxygenasaes (ClcA, TfdC, TcbC) is between 53.5 and 63%, whereas the a.a. sequence homology between the chlorocatechol 1,2-dioxygenases and the catechol 1,2-dioxygenases (CatA and the EST1001 plasmid encoded PheB) is below 25%. CatA and PheB are closely related having a 57% a.a. sequence homology (Ghosal and You 1988; van der Meer et al. 1991b).

The cycloisomerases TcbD, ClcB, TfdD, and CatB are more conserved than the catechol 1,2-dioxygenases, based on a.a. sequence comparison. TcbD and CatB were found to be 43.9 % identical and all the cycloisomerases contained 370 amino acid residues (van der Meer et al. 1991b). Comparison of the cycloisomerases, *clcB* and *tfdD* at the nucleotide level revealed 60% or higher homology (Ghosal and You 1988). The three hydrolases encoded by the *tcbE*, *clcD* and *tfdE* genes showed 52% homology.

2.3 Uptake of aromatic and chloro-aromatic compounds

Uptake is generally considered to be the first step in the degradation pathway of most substrates. However, little is known concerning the uptake mechanisms of aromatic and chlorinated aromatic compounds. Uptake of aromatic compounds has been observed from two different perspectives. Firstly, the uptake of non-metabolizable aromatic compounds normally entails the evolution of physiological and structural modifications in the organism which results in restricted permeation. The controlled membrane permeation of toxic compounds is obviously important to microorganisms unable to degrade these compounds. Secondly, the uptake of metabolizable aromatic compounds can occur by diffusion or via the evolution of highly specific permeases.

Lipophilic weak acids have been assumed to traverse cell membranes by passive diffusion. These compounds have been used for measuring pH gradients across membranes (ΔpH) (Kihara and Macnab 1981; Kashket 1985). Uptake of benzoic acid and aliphatic weak acids by yeasts have been studied for the purpose of understanding the preservative properties of these compounds added to acidic foods and beverages. The rate of benzoic acid uptake by the yeast *Zygosaccharomyces bailii* is proportional to the concentration of the undissociated form of the acid (Warth 1989). The inhibitory action of benzoic acid at low pH's is due mainly to the energy demand required for maintaining both the intracellular pH alkaline and the intracellular concentration of the dissociated acid low. Tolerance to benzoic acid by *Zygosaccharomyces bailii* cells is acquired by reducing the permeability of the cytoplasmic membrane to benzoic acid (Warth 1989). Similarly, the uptake of 2,4-D by *Pseudomonas fluorescens* markedly increased as the pH of the suspending medium was lowered below 6. This compound, which is not metabolized by *Pseudomonas fluorescens*, was not concentrated intracellularly

against a gradient and uptake was inhibited by the respiratory inhibitor, sodium azide. These observations suggest that the undissociated form of 2,4-D enters via diffusion through the membrane down a concentration gradient and in response to an intracellular alkaline environment (Wedemeyer 1966). *Escherichia coli* acquired tolerance to phenol by synthesizing phospholipids containing predominantly saturated fatty acids which reduces the permeability of the membrane to the toxic compound (Keweloh et al. 1991). This controlled permeation of toxic substances also occurs in microorganisms which are capable of utilizing these substances as sole carbon sources. Growth of *Pseudomonas putida* P8 on phenol and 4-chlorophenol resulted in an increase of the saturated to unsaturated fatty acid ratio and a conversion of *cis* to *trans* unsaturated fatty acids. Both mechanisms are believed to decrease the permeability of the cytoplasmic membrane to phenol. Intracellular accumulation of the phenolic compounds against a concentration gradient was not addressed (Heipieper et al. 1992).

The role of permeases (stereospecific transmembrane proteins) has been implicated in the uptake of benzoic acid and 4-CBA, based on observed uptake saturation kinetics (Thayer and Wheelis 1982; Harwood and Gibson 1986; Groenewegen et al. 1990). However, unlike the well characterized transport systems of amino acids and sugars, the alleged permeases involved in the transport of aromatic compounds have not been characterized.

The metabolic activity of the cells is of crucial importance when interpreting data obtained from transport assays of metabolizable substrates. The simple detection of radioactivity in the cells does not differentiate between a labeled substrate in an unmodified state inside the cell and metabolic products derived from the labeled substrate. In the absence of such information, the definite nature of an uptake system can not be ascertained. A case in point is

the uptake of benzoic acid and 4-HBA under anaerobic conditions by *Rhodopseudomonas palustris* (Harwood and Gibson 1986; Merkel et al. 1989). Uptake of these substrates was saturable and was completely inhibited in assays carried out in darkness or in the presence of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Kinetic analyses of the uptake data revealed high affinities for benzoic acid (K_m below 1 μM) and 4-HBA (K_m of 0.3 μM). These preliminary results were indicative of an uptake other than passive diffusion. However extraction and analysis of the intracellular labeled products revealed benzoyl CoA as the major radioactive component. The CoA synthetase activity, which is the first degradative step in the anaerobic degradation of benzoic acid by *Rhodopseudomonas palustris* (Harwood and Gibson 1986), is very rapid and results in the conversion of benzoic acid and 4-HBA to benzoyl CoA and 4-hydroxybenzoyl CoA, respectively, immediately upon entry of the substrate into the cytoplasm. It is thus not clear whether benzoic acid and 4-HBA enter the cell by carrier mediated transport or by passive diffusion. Chemical conversion (thioesterification) of the substrate upon entry into the cytoplasm serves to maintain a downhill concentration gradient between the cytoplasm and the extracellular medium. Energy is required for the metabolism of the substrate and thus indirectly for the uptake as well. Therefore, the apparent saturation kinetics and high affinity uptake may be due to the rapid conversion of the free acids to their CoA derivatives by the CoA ligases, since cells lacking the CoA synthetase enzyme (succinic acid grown cells) did not take up either substrate. The role of facilitated diffusion or uptake driven by thioesterification of the substrates via group translocation remains a possibility. However, the existence of permease or membrane-bound thioesterification activities was not forthcoming (Harwood and Gibson 1986; Merkel et al. 1989). The above

mentioned uptake of benzoic acid and 4-HBA exemplifies some of the shortcomings that can be experienced when studying uptake of metabolizable substrates by metabolically active cells. Whenever possible, the use of non-metabolizable substrate analogues and/or non-metabolizing membrane vesicles is recommended, although these approaches also have inherent limitations. Reliable estimates of intracellular solute concentrations under conditions of active uptake using membrane vesicles, may be difficult (Hamilton 1975). Also, in the absence of any information concerning the role of binding proteins in the uptake of aromatic and chloroaromatic substrates, the use of vesicles is not recommended since inherent in their preparation is the loss or alteration of binding proteins (Hamilton 1975). Mutants defective in the subsequent metabolism of the test substrate have been used in determining active uptake of benzoic acid, 4-HBA and 3,4-DHBA (Thayer and Wheelis 1982; Wong et al. 1991).

Active transport is an energy-dependent process whereby unmodified substrate is accumulated so that its concentration inside the cell is greater than in the extracellular medium (Hamilton 1975). Simple and facilitated diffusion merely result in equilibration of the substrate across the cell membrane and do not require any input of energy. Facilitated diffusion unlike simple diffusion, exhibits saturation uptake kinetics since it relies on a fixed number of stereospecific transmembrane proteins that allow specific compounds to diffuse through the membrane (Hamilton 1975). Thayer and Wheelis (1982) measured a 150-fold benzoic acid accumulation against a concentration gradient using a mutant strain of *Pseudomonas putida* unable to metabolize benzoic acid. The inducible and saturable benzoic acid uptake was stimulated by a respirable carbon source, succinic acid, and inhibited by azide (cytochrome oxidase inhibitor), CCCP (carbonyl cyanide m-chlorophenylhydrazone, a protonophore),

arsenate (an analogue of phosphate and hence an inhibitor of high energy phosphate bond formation), and DCCD (dicyclohexylcarbodiimide, which blocks the proton channel in the Fo subunit of the ATPase). Initial uptake rates did not correlate with the cell's ATP pool levels. These observations suggest an active uptake of benzoic acid into *P. putida* energized by a proton motive force (Δp). The component of the proton motive force driving the transport, was not determined. The exact nature of the mutation was not determined, although it was not at the level of the benzoic acid dioxygenase. Since the 150-fold intracellular accumulation was based solely on the accumulated intracellular radioactivity and no extraction and identification of this metabolite was performed, transformation of benzoic acid could have occurred upon entry into the cytoplasm.

A recent study on the uptake of hydroxy-aromatic compounds by *Rhizobium leguminosarum*, revealed the existence of two distinct uptake systems for 4-HBA and 3,4-DHBA (Wong et al. 1991). The enzyme, 4-hydroxy benzoic hydroxylase, converts 4-HBA to 3,4-DHBA which is subsequently dioxygenated via a protocatechuic acid 3,4-dioxygenase. Attempts to obtain 4-hydroxybenzoic acid hydroxylase mutants or any other mutation preventing the metabolism of 4-HBA were unsuccessful. Consequently, uptake studies were carried out with wild-type strains. Cells grown on 4-HBA took up 4-HBA and 3,4-DHBA. Cells grown on 3,4-DHBA took up 3,4-DHBA at the same rate as 4-HBA grown cells and 4-HBA at a rate of only 10% of cells grown on 4-HBA. Uptake of these substrates was inhibited by CCCP and 2,4-dinitrophenol (DNP). Unlike benzoic acid uptake by *P. putida*, an alternate respirable carbon source did not stimulate uptake rates. However, root nodule bacteria accumulate poly β-hydroxybutyrate which can presumably fuel solute uptake (Wong et al. 1991). These results suggest the possibility that *R. leguminosarum*

may have separate uptake systems for 4-HBA and 3,4-DHBA. Although mutants defective in the metabolism of 4-HBA were not isolated, a mutant which contained the full complement of enzymes required for the metabolism of 4-HBA but which did not take up this substrate was obtained. This mutant was believed to be 4-HBA permease-defective based on the fact that it transported and metabolized 3,4-DHBA, the next intermediate in the pathway, when grown on glucose supplemented with either 4-HBA or 3,4-DHBA. This also suggested the existence of a distinct carrier for 3,4-DHBA which was further confirmed by the isolation of mutants lacking protocatechuate 3,4-dioxygenase which meant that it was possible to measure [^{14}C] 3,4-DHBA in the absence of metabolism. These mutants accumulated 3,4-DHBA against a 78-116 fold concentration gradient and is evidence of an active uptake system. Inhibition by metabolic inhibitors was not sufficiently definitive to suggest the nature of the uptake driving force. The effect of substrate analogues (50-fold molar excess) on uptake of 4-HBA and 3,4-DHBA by 4-HBA grown cells indicated that the protocatechuic acid uptake system was completely inhibited by other dihydroxybenzoic acids, whereas, it was slightly inhibited by 4-HBA. The 4-HBA uptake system was not greatly affected by dihydroxybenzoic acids with the exception of 3,4-DHBA which totally inhibited 4-HBA uptake.

The uptake of 4-CBA by the coryneform bacterium NTB-1 is the only documented carrier-mediated transport of halogenated aromatic compounds in bacteria. The degradation of 4-CBA by this organism, discussed in section 2.2.1.2.2, proceeds by initial hydrolytic dehalogenation forming 4-HBA. This dehalogenation can occur anaerobically, but the subsequent metabolism of 4-HBA mandates aerobic conditions (Groenewegen et al. 1990). Uptake of 4-CBA is inducible and exhibited saturation kinetics with a high apparent affinity (K_m 1.7 μM). Uptake and respiration of 4-CBA occurred simultaneously under

aerobic conditions. Anaerobically, 4-CBA uptake was not observed. However, addition of nitrate transiently restored 4-CBA uptake. Under these conditions a steady-state was reached corresponding to a 30-fold accumulation of 4-HBA, which in the absence of oxygen was subsequently excreted into the suspending medium. This does not entirely rule out a passive diffusion mechanism followed by metabolism. The authors suggested that the inability of the cells to transport 4-CBA anaerobically was due to the imposition of a low proton motive force (Δp) under these conditions. Consequently, they proceeded to investigate the role of the Δp on 4-CBA uptake under anaerobic conditions at pH 7.0. The Δp consists of two components, a concentration gradient of protons (Δ pH) and an electrical potential or membrane potential ($\Delta \psi$), across the membrane. An artificially imposed Δp was set up as follows:

(i) a Δ pH, inside alkaline was generated by diluting K+ and acetate loaded cells in the presence of valinomycin into a buffer containing potassium phosphate and a less permeable anion, such as 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid) (HEPES).

(ii) a $\Delta \psi$, inside negative was created by diluting K+ and acetate loaded cells in sodium phosphate and sodium acetate buffer in the presence of valinomycin, a mobile carrier ionophore which catalyzes the electrical uniport of K+, Cs+, Rb+ or NH4+.

(iii) a Δp, inside negative and alkaline was established by diluting K+ and acetic acid loaded cells in a solution containing sodium phosphate and HEPES buffer in the presence of valinomycin. This imposes an outward diffusion gradient of K+ ions and acetate.

Cells with no Δp (K^+ and acetate loaded cells diluted in a buffer containing potassium phosphate and potassium acetate in the presence of valinomycin) failed to transport 4-CBA. Uptake occurred in cells with a Δ pH and to a lesser extent in cells with a $\Delta \psi$. The highest level of uptake occurred in cells when both a Δ pH and a $\Delta \psi$ were imposed simultaneously. In an attempt to further dissect the role of the Δp, the effect of the ionophores, valinomycin and nigericin, on 4-CBA uptake under aerobic conditions was investigated. The effect of these two ionophores was shown to be pH dependent. Nigericin, which dissipates the Δ pH in the presence of K^+ ions by mediating electroneutral H^+-K^+ exchange, inhibited uptake at pH 5.0. Inhibition diminished with increasing pH. Valinomycin, which dissipates the $\Delta \psi$ in the presence of K^+ ions, was effective only at pH 7 and above. The simultaneous addition of nigericin and valinomycin, which abolishes the Δp, completely inhibited uptake at pH 5 but was not as effective at neutral and alkaline pH. These observations in conjunction with the uptake results of artificially imposed Δp conditions suggest that at low pH, uptake of 4-CBA is coupled to the Δ pH whereas at alkaline pH, uptake is coupled to $\Delta \psi$.

Inhibition studies with structurally related compounds (30-fold molar excess) suggested a 4-CBA uptake system with specificity for *para*-substituted mono-halobenzoic acid and for 2,4- and 3,4-DCBA. Benzoic acid inhibited 4-CBA uptake, albeit poorly. The nature of the inhibition was not determined and thus, whether or not these analogues are actively transported is not known. It is important to remember that all observations in this study were obtained with metabolically active cells and with a metabolizable substrate. Whether or not 4-CBA was accumulated against a concentration gradient, was not determined.

Section 3

Degradation of mono- and dichlorobenzoic acid isomers by two natural isolates of *Alcaligenes denitrificans.*

3.0 Preface

This section describes the enrichment, isolation, identification and initial characterization of chlorobenzoic acid degraders from PCB contaminated soil. The range of aromatic compounds metabolized by the isolated microorganisms, and the mechanism by which the aromatic ring is cleaved, were determined. This section is drawn from the publication:

Miguez, C.B., C.W. Greer, and J.M. Ingram. 1990. Degradation of mono- and dichlorobenzoic acid isomers by two natural isolates of *Alcaligenes denitrificans.* Arch Microbiol 154:139-143.

3.1 Abstract

Two strains of *Alcaligenes denitrificans*, designated BRI 3010 and BRI 6011, were isolated from polychlorinated biphenyl (PCB) contaminated soil using 2,5-dichlorobenzoic acid (2,5-DCBA) and 2,4-DCBA, respectively, as sole carbon and energy sources. Both strains degraded 2-chlorobenzoic acid (2-CBA), 2,3-DCBA, 2,5-DCBA, and were unable to degrade 2,6-DCBA. BRI 6011 alone degraded 2,4-DCBA. Growth of BRI 6011 in yeast extract and 2,6-DCBA induced pyrocatechase activity, but 2,6-DCBA was not degraded, suggesting the importance of an unsubstituted carbon six of the aromatic ring. Metabolism of the chlorinated substrates resulted in the stoichiometric release of chloride, and degradation proceeded by intradiol cleavage of the aromatic ring. Growth of both strains on 2,5-DCBA induced pyrocatechase activities having catechol and chlorocatechols as substrates. In contrast to dichlorobenzoic acids, growth on 2-CBA, benzoic acid, mono- and dihydroxybenzoic acids induced a pyrocatechase activity directed against catechol only. Although 2,4-DCBA was a more potent inducer of both pyrocatechase activities, its utilization by BRI 6011 was inhibited by 2,5-DCBA. Specific uptake rates using resting cells were highest with 2-CBA, except when the resting cells had been previously grown on 2,5-DCBA, in which case 2,5-DCBA was the preferred substrate. The higher rates of 2,5-DCBA uptake obtained by growth on that substrate, suggested the existence of a separately induced uptake system for 2,5-DCBA.

Key Words: Aromatic compounds - 2-chlorobenzoic acid - Dichlorobenzoic acids - Degradation - Pyrocatechase - Uptake - *Alcaligenes denitrificans*

3.2 Introduction

Chlorobenzoic acids are present in the environment due to the widespread use of herbicides, and as products of the partial microbial degradation of polychlorinated biphenyls (PCBs). Consequently, microorganisms capable of metabolizing chlorobenzoic acids are essential for the complete mineralization of these recalcitrant compounds. Published reports reveal the large number of genera capable of utilizing chlorobenzoic acids as sole sources of carbon and energy. The metabolism of chlorobenzoic acids can be initiated by hydrolytic (Marks et al.1984a, 1984b; Muller et al.1984; van den Tweel et al.1987) or reductive (Dolfing and Tiedge 1987; van den Tweel et al. 1987) dehalogenation, or more commonly, the majority of aerobic microorganisms degrade these substrates by non-specific enzyme systems that do not directly affect the carbon-halogen bond. Ring cleavage of chlorocatechols, which are intermediates in this pathway, is catalyzed by non-specific catechol 1,2-dioxygenases and dechlorination occurs at a later stage in the pathway (Ngai and Ornston 1988). Also interesting amongst chlorobenzoic acid metabolizers, regardless of the dehalogenation mechanism, is the narrow range of chlorobenzoic acid isomers that can serve as substrates (Keil et al.1981; Grishchenkov et al.1983; Engesser and Schulte 1989). Microorganisms that metabolize a wide range of chlorobenzoic acid isomers are far less abundant (Hartmann et al.1979; Baggi 1985; Hickey and Focht 1989). In the present study two strains of *Alcaligenes denitrificans*, BRI 3010 and BRI 6011, were isolated from a chemostat enrichment culture of PCB contaminated soil using 2,5-DCBA (3 mM) and 2,4-DCBA (3 mM), respectively, as sole carbon sources. The purpose of this study was to characterize the two strains and elucidate the substrate range as well as the rate of metabolism of chlorobenzoic acids.

3.3 Materials and Methods

Organisms. Strains BRI 3010 and BRI 6011 were isolated from an environmental soil sample contaminated with PCB (Aroclor 1254, 800 ppm) from Smith Falls, Ontario, by enrichment culture using a minimal salts medium (MSM) containing 3 mM 2,5-DCBA and 3 mM 2,4-DCBA, respectively, as sole carbon and energy sources. The organisms were identified as *Alcaligenes denitrificans* using Rapid NFT strips (API Systems, S.A.,France) and the GN Microplate (Biolog, Inc., Hayward, CA). Additional standard tests (Gram stain, motility, etc.) were performed by standardized procedures (Doetsch 1981).

Growth media. The minimal salts medium (MSM) was composed as follows (g/l); KH_2PO_4, 0.87; K_2HPO_4, 2.26; $(NH_4)_2SO_4$, 1.1; and $MgSO_4.7H_2O$, 0.097. To this solution was added 1 ml (per litre) of a trace metals solution composed of (g/l): $Co(NO_3)_2.6H_2O$, 0.291; $AlK(SO_4)_2.12H_2O$, 0.474; $CuSO_4$, 0.160; $ZnSO_4.7H_2O$, 0.288; $FeSO_4.7H_2O$, 2.78; $MnSO_4.H_2O$, $Na_2MoO_4.2H_2O$, 0.482; and $Ca(NO_3)_2.4H_2O$, 2.362. The final pH was 7.2. The aromatic growth substrates were used at concentrations of 0.5 or 1.0 mM, as indicated. Hydroxybenzoic acids were added from a filter sterilized stock solution (100 mM). Whenever required, yeast extract (Y.E.) (0.1 or 5 g/l) was included in the medium.

Growth studies. Growth experiments were conducted in either 500 or 1000 ml Erlenmeyer flasks. The volume ratio between the growth medium and the flask was maintained constant (1:5). All flasks were incubated at 30^{0}C, and 250 rpm (G24, New Brunswick Sci. Co.). At the indicated sampling times, 2.5 ml of culture was removed for optical density (660 nm) measurements and following centrifugation (Eppendorf), for analysis of supernatant, for substrate by HPLC (1.2 ml), and for chloride (0.3 ml). Experiments to ascertain the range of aromatic compounds utilized by BRI 3010 and 6011 were performed in 100 ml

of 1 mM substrate inoculated with 2,5-DCBA grown cells. For phenol and chlorinated phenols, a concentration of 0.1 mM was used. Samples were taken daily for a maximum duration of 16 days, and analyzed as described below.

Preparation of resting cells. Cells grown in 500 ml MSM containing 0.1 g/l Y.E. and 0.5 mM of the respective chlorobenzoic acid were harvested by centrifugation (10,000 x g, 20 min, 4^0C) in late exponential growth phase, washed twice with sterile MSM, and subsequently resuspended in sterile MSM to yield an optical density (660 nm) of 0.1 (30-35 ug/ml cell protein) for the substrate uptake assay.

Substrate uptake assay. Resting cells of BRI 3010 and BRI 6011 were used to determine the rate of mono- and dichlorobenzoic acid uptake. The reaction mixture, consisting of resuspended cells (30-35 ug/ml total protein) in MSM (30 ml), was incubated at 30^0C, 250 rpm for 25 min prior to the addition of substrate. At time 0, 0.3 ml of a sterile stock solution (10 mM) in MSM of mono- or dichlorobenzoic acid was added. At times 0, 1.5 min, and at 4 min intervals for 40 min, 0.95 ml aliquots were removed from the reaction mixture and immediately centrifuged (Eppendorf). The supernatant was transferred into HPLC vials containing 0.02 ml 1 N H_2SO_4 and frozen until analyzed. Cells boiled for 10 minutes were used as negative controls. Uptake rates were determined from the initial linear portion of the rate curve, and replicate assays showed less than 5% variation in uptake rates.

Preparation of cell extracts. Crude cell extracts were prepared as follows: Cells were grown in 500 ml MSM containing 5 g/l Y.E. and 1 mM of the corresponding aromatic substrate at 30^0C, and 250 rpm. The culture was harvested at late exponential growth phase, centrifuged (10,000 x g, 20 min, 4^0C), washed twice with sterile MSM, resuspended in 10 ml TEB buffer (50 mM

TRIS/sulfate, 1 mM EDTA, 1 mM B-mercaptoethanol, pH 7.5.), and cells were disrupted by sonication (model W-375, Heat Systems-Ultrasonics, Inc.). Cell debris was removed by centrifugation (20,000 x g, 20 min, 4^0C), and the resulting crude extract was assayed for pyrocatechase activity.

Pyrocatechase assay. Pyrocatechase was assayed in a reaction mixture composed of 950 ul TEβ buffer, 40 ul catechol (Aldrich Chemical Company, Inc., Milwaukee, USA.) or substituted catechol (Helix Biotech Ltd. Vancouver, Canada.) stock (10 mM) distilled water, and 10 ul of crude cell extract (5-10 ug protein/ul). The reaction was started by the addition of substrate. Enzyme activity was assayed spectrophotometrically (model DU-7,Beckman) by measuring the rate of cis,cis-muconic acid (260 nm) or substituted cis,cis-muconic acid formation. To calculate enzyme activities, the molar absorption coefficients of substituted muconates (Dorn and Knackmuss 1978b) were used. 4-chlorocatechol (4-CC), and 3,5-dichlorocatechol (3,5-DCC) were tested as chlorinated catechols because they are potential metabolites of 2,4-DCBA degradation, and both were commercially available. One unit of enzyme activity is defined as that amount which catalyzes the formation of 1 micromole of product per minute.

Metapyrocatechase assay. Enzyme activity was assayed spectrophotometrically as described by Nozaki (1970), by measuring the rate of 2-hydroxymuconic acid semialdehyde formation (375 nm).

Analytical. Benzoic acid, mono- and dichlorobenzoic acids, were determined by HPLC (Spectra-Physics, SP8800) using a 25 cm ODS-1 (octadecylsilane) column at 55^0C, with methanol:acetic acid (55:45 of 0.1%) as solvent at a flow rate of 0.7 ml/min. Mono- and dihydroxybenzoic acids were determined as above, except that the solvent ratio was (70:30 of 0.1%). Detection was by U.V. at 210 nm. Phenol, and chlorophenols were analyzed as

above, but the mobile phase consisted of an increasing methanol, decreasing KH_2PO_4 (0.02 M, pH 4) gradient (55 methanol:45 KH_2PO_4 to 67 methanol:33 KH_2PO_4) at a flow rate of 0.7 ml/min.

Inorganic chloride was determined colorimetrically (Florence and Farrar 1971), using a sample volume of 1.0 ml, and sodium chloride as standard.

The Lowry method (Markwell et al.1978), was used to estimate protein concentrations with bovine serum albumin as standard.

3.4 Results

Identification of isolates. BRI 3010 and BRI 6011 were Gram negative, oxidase positive, motile short rods. They were characterized as *Alcaligenes denitrificans* using Rapid NFT strips and the GN Microplate. Both isolates possessed virtually identical metabolic capabilities with two exceptions, and grew on the following carbon sources: acetic acid, L-aspartic acid, citric acid, formic acid, L-glutamic acid, D-gluconic acid, *cis*-aconitic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, L-pyroglutamic acid, propionic acid, D,L-lactic acid, D-saccharic acid, succinamic acid, succinic acid, α-keto-glutaric acid, urocanic acid, L-alanyl-glycine, L-proline, L-alanine, alaninamide, methyl pyruvate, and bromosuccinic acid. In addition to these substrates, BRI 6011 also metabolized D-alanine.

Utilization of substituted benzoic acids and phenols by *A. denitrificans* BRI 3010 and BRI 6011 . A variety of substituted benzoic acids and phenols were assayed to ascertain the range of substrates that could be utilized by the isolates as sole carbon sources (Table 3.1). The only difference observed in the substrate utilization profile of the two strains was the ability of BRI 6011 to utilize 2,4-DCBA. All mono- and di- chlorobenzoic acids with chlorine in the 2-position, (2-CBA, 2,3-DCBA, 2,4-DCBA, and 2,5-DCBA) were degraded, with the exception of 2,6-DCBA. The complete degradation of 0.5 mM 2,5-DCBA by BRI 3010 occurred within 31 hours of growth (Fig.3.1). Chloride release was stoichiometric and mirrored the disappearance of substrate in the medium. The inclusion of Y.E. (100 mg/l) in the growth medium resulted in the complete utilization of 2,5-DCBA within 14 hours.

Uptake of mono- and dichlorobenzoic acids by BRI 3010 and BRI 6011 resting cells. BRI 3010 and BRI 6011 demonstrated differential uptake rates for the metabolizable chlorobenzoic acids (Table 3.2). Uptake of

Table 3.1 Utilization of aromatic compounds as sole carbon sources by BRI 3010 and BRI 6011

Substrate	Utilization[a]	
	BRI 3010	BRI 6011
benzoic acid	+	+
2-hydroxybenzoic acid	+	+
3-hydroxybenzoic acid	+	+
4-hydroxybenzoic acid	-	-
2,3-dihydroxybenzoic acid	-	-
2,4-dihydroxybenzoic acid	-	-
2,5-dihydroxybenzoic acid	+	+
2,6-dihydroxybenzoic acid	-	-
3,4-dihydroxybenzoic acid	+	+
3,5-dihydroxybenzoic acid	-	-
2-chlorobenzoic acid	+	+
3-chlorobenzoic acid	-	-
4-chlorobenzoic acid	-	-
2,3-dichlorobenzoic acid	+	+
2,4-dichlorobenzoic acid	-	+
2,5-dichlorobenzoic acid	+	+
2,6-dichlorobenzoic acid	-	-
3,4-dichlorobenzoic acid	-	-
3,5-dichlorobenzoic acid	-	-
phenol	-	-
2,4-dichlorophenol	-	-
2,5-dichlorophenol	-	-

[a] Utilization was based on the disappearance of substrate by HPLC, and an increase in inorganic chloride (where applicable)

Figure 3.1

Growth of BRI 3010 in 0.5 mM 2,5-DCBA. Growth was monitored by optical density at 660 nm (O.D. 660 nm) (●), and 2,5-DCBA degradation was monitored by HPLC (▲) and by the appearance of chloride (■).

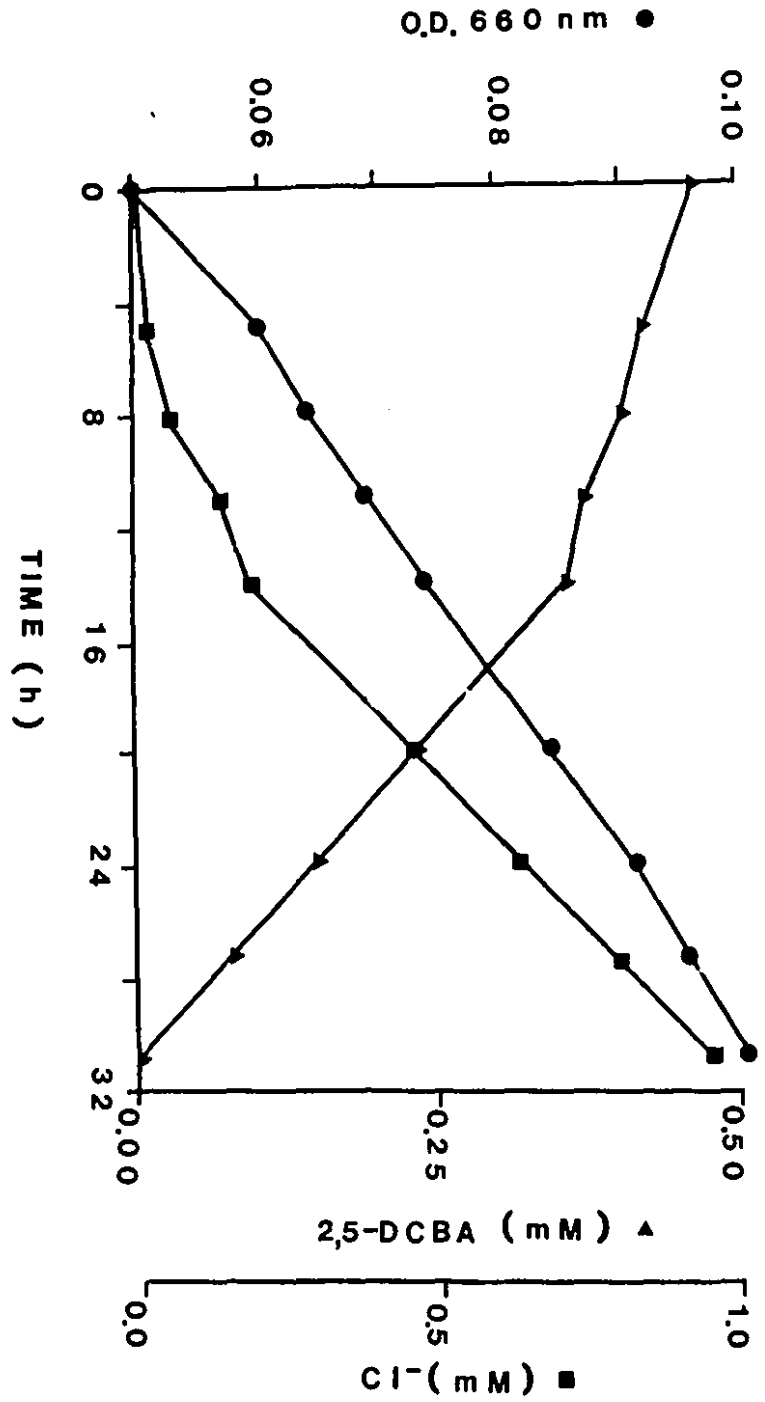

O.D. 660 nm ●
0.06
0.08
0.10
0
8
16
24
32
TIME (h)
0.00
0.25
0.50
2,5-DCBA (mM) ▲
0.0
0.5
1.0
Cl⁻ (mM) ■

Table 3.2 Substrate uptake responses of resting cells of *A. denitrificans* BRI 3010 and BRI 6011 to chlorobenzoic acids

Strain	Growth substrate	Substrate uptake rate* (μmol/min/mg protein) with test substrates:			
		2-CBA	2,3-DCBA	2,4-DCBA	2,5-DCBA
BRI 3010	2-CBA	0.079	0.005	-	0.052
	2,5-DCBA	0.083	0.006	-	0.122
BRI 6011	2-CBA	0.098	0.015	0.029	0.041
	2,4-DCBA	0.064	0.009	0.017	0.048
	2,5-DCBA	0.084	0.010	0.016	0.155

For assay conditions see Materials and Methods.

*Replicate assays showed less than 5% variation in uptake rates

2-CBA, 2,3-DCBA, and 2,4-DCBA was relatively independent of the growth substrate. Uptake of 2,5-DCBA increased several fold if cultures were grown on that substrate, although uptake of 2,5-DCBA was possible independent of chlorobenzoic acid growth substrate.

Substrate competition studies with actively growing cells. When BRI 6011 was pre-grown on 2,5-DCBA alone, and subsequently allowed to grow in both 2,4-DCBA and 2,5-DCBA (0.5 mM each), there was essentially no uptake of 2,4-DCBA until 2,5-DCBA in the growth medium had been depleted (Fig. 3.2). Alternatively, if the cells had been pre-grown on 2,4-DCBA, were actively growing on 2,4-DCBA, and were spiked with 2,5-DCBA, inhibition of 2,4-DCBA uptake was observed (Fig. 3.3). When 2,5-DCBA and 2,4-DCBA were mixed at a ratio of 1:5, 2,5-DCBA remained the preferred substrate (Fig. 3.4). Growth substrate dependent uptake of 2,4-DCBA and 2,5-DCBA is also evident in Table 3.2, whereby cells grown on 2,5-DCBA took up that substrate at approximately three times the rate of cells grown on 2,4-DCBA (Table 3.2). Uptake of 2,4-DCBA remained the same regardless of growth substrate.

Pyrocatechase activity of cell extracts. Degradation of substituted benzoic acids by BRI 3010 and BRI 6011 proceeded by intradiol cleavage of the aromatic ring. No catechol 2,3-dioxygenase (metapyrocatechase) was detected. Growth of the cultures on dichlorobenzoic acids induced catechol 1,2-dioxygenase (pyrocatechase) as determined by specific activities against catechol and chlorocatechols (Table 3.3). Catechol, 4-chlorocatechol (4-CC) and 3,5-dichlorocatechol (3,5-DCC) served as substrates for pyrocatechase, but the cell extract was unable to react with 3,4-DCC, or 4,5-DCC (data not shown). Growth of BRI 3010 and BRI 6011 on 2,5-DCBA induced a pyrocatechase activity that exhibited similar specificities against all catechol substrates, whereas growth on 2-CBA induced pyrocatechase with activity directed against

Figure 3.2

Growth of BRI 6011 in 0.5 mM 2,5-DCBA (▲) and 0.5 mM 2,4-DCBA (■) supplemented with 100 mg/l yeast extract. Inoculum was grown on yeast extract supplemented 2,5-DCBA. Growth was monitored by optical density at 660 nm (O.D. 660 nm) (●). Utilization of dichlorobenzoic acids was determined by HPLC.

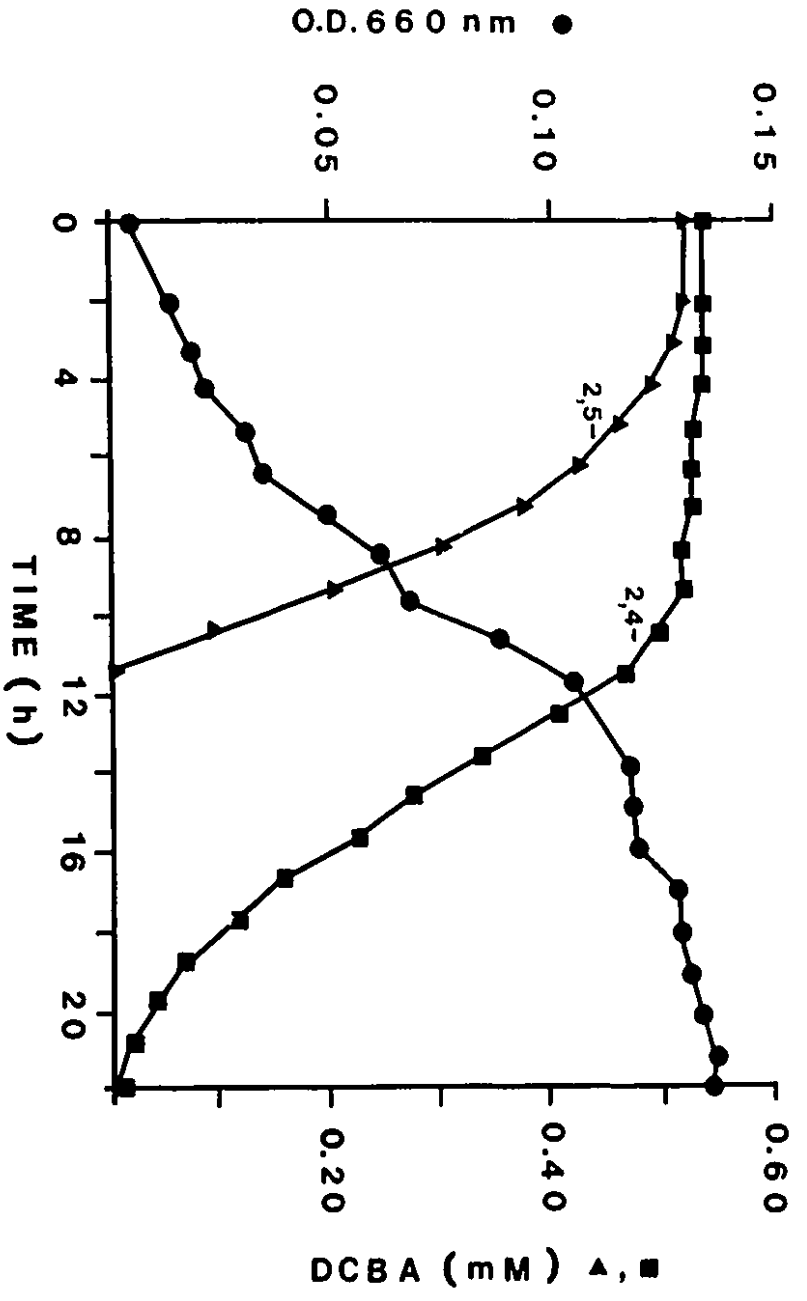

O.D. 660 nm ●
0.05
0.10
0.15
0
4
8
12
16
20
TIME (h)
2,5-
2,4-
0.20
0.40
0.60
DCBA (mM) ▲, ■

Figure 3.3

Effect of 2,5-DCBA (0.5 mM) (▲) addition to a culture of BRI 6011 growing on 2,4-DCBA (0.5 mM) (■) supplemented with yeast extract. Inoculum was grown on yeast extract supplemented 2,4-DCBA. Growth was followed by optical density at 660 nm (O.D. 660 nmm) (●). Utilization of dichlorobenzoic acids was monitored by HPLC.

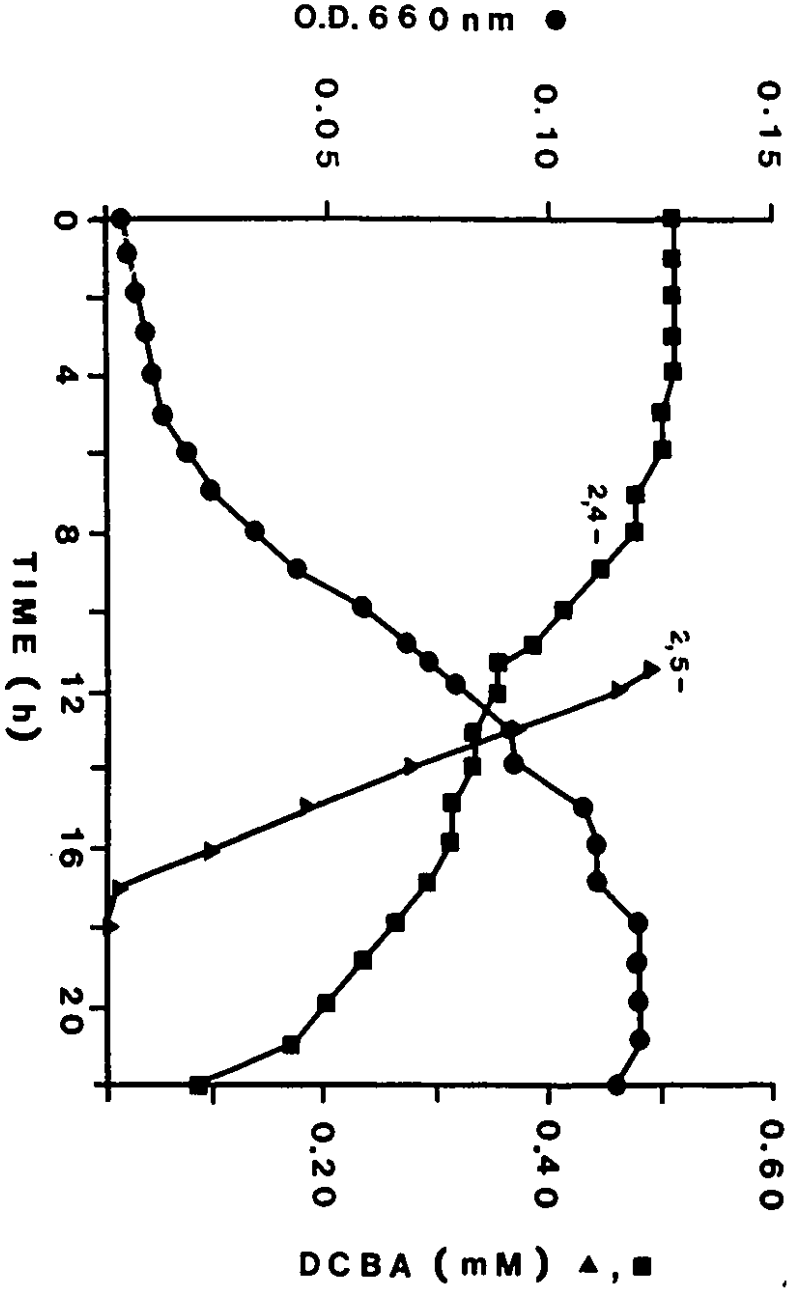

O.D. 660 nm ●
0.05
0.10
0.15
0
4
8
12
16
20
TIME (h)
2,4-
2,5-
0.20
0.40
0.60
DCBA (mM) ▲, ■

Figure 3.4

Growth of BRI 6011 in 0.1 mM 2,5-DCBA (▲) and 0.5 mM 2,4-DCBA (■) supplemented with 100 mg/l yeast extract. Inoculum was grown on yeast extract supplemented 2,5-DCBA. Growth was monitored by optical density at 660 nm (O.D. 660 nm) (●). Utilization of dichlorobenzoic acids was determined by HPLC.

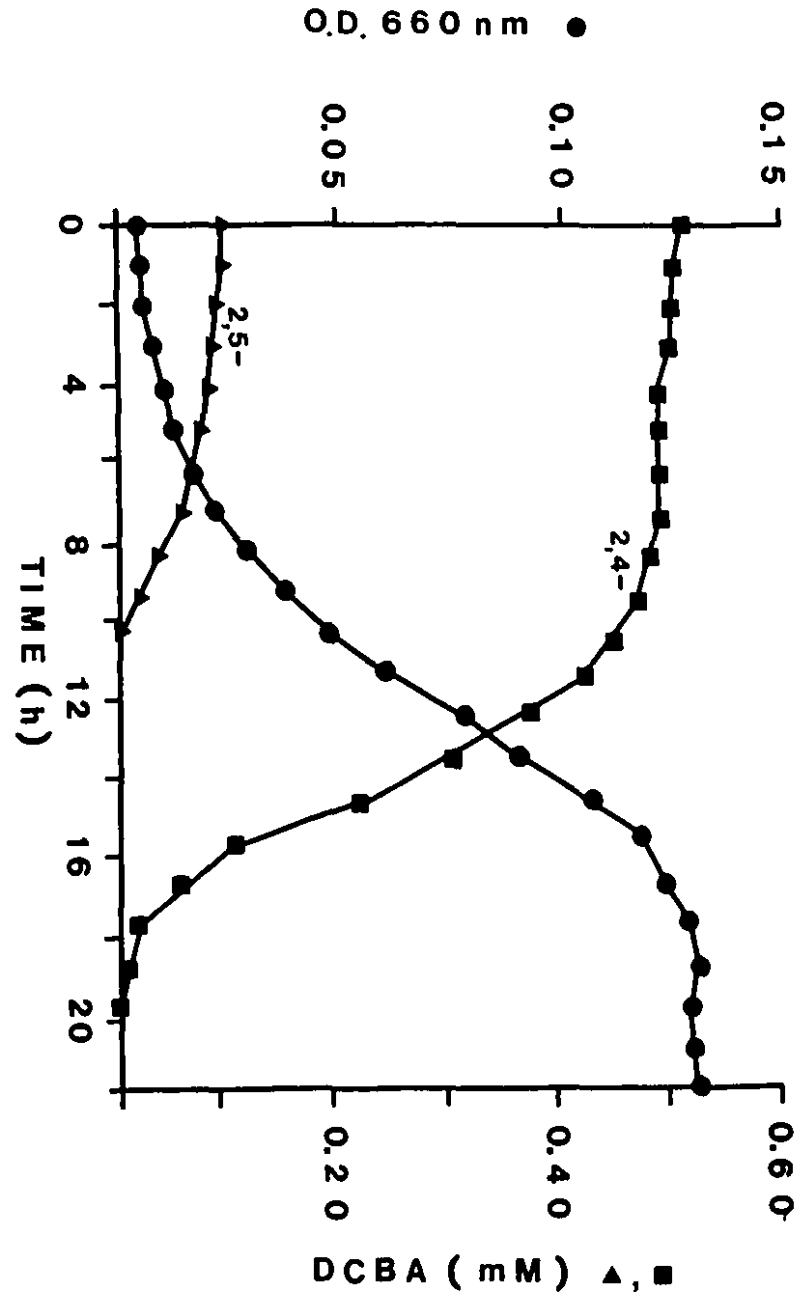

O.D. 660 nm ●
0.05
0.10
0.15
0
4
8
12
16
20
TIME (h)
2,5-
2,4-
0.20
0.40
0.60
DCBA (mM) ▲, ■

Table 3.3 Specific activities of pyrocatechases in cell extracts of *A. denitrificans* BRI 3010 and BRI 6011

Strain	Growth substrate	Specific activity (mU/mg protein) for substrates:		
		Catechol	4-CC	3,5-DCC
BRI 3010	2,5-DCBA	51(1)	41(1)	59(1)
	2-CBA	55(1)	13(0)	6(1)
	Y.E	8(1)	6(1)	4(0)
BRI 6011	2,4-DCBA	129(5)	107(5)	95(3)
	2,5-DCBA	49(4)	62(3)	53(0)
	2,6-DCBA*	38(2)	15(1)	9(2)
	2-CBA	49(2)	12(1)	3(1)
	B.A.	45(2)	9(1)	6(1)
	Y.E.	10(2)	9(1)	4(1)

(Y.E.) Yeast Extract
(B.A.) Benzoic acid;
(4-CC) 4-chlorocatechol
(3,5-DCC) 3,5-dichlorocatechol
For assay conditions see Materials and Methods.
*Substrate not utilized by the culture
( ) Standard deviation of triplicate assays

catechol only (Table 3.3). The specific activities obtained with 2-CBA as a growth substrate were comparable to those obtained with benzoic acid (Table 3.3), and hydroxybenzoic acids (data not shown). Growth of BRI 6011 on 2,4-DCBA resulted in significantly higher levels of pyrocatechase activity (Table 3.3). BRI 6011 was unable to metabolize 2,6-DCBA, but pyrocatechase activity directed against catechol was induced (Table 3.3). The differential induction of pyrocatechase activities directed against catechol during growth on benzoic acid or 2-CBA, and activities directed against both catechol and chlorocatechol during growth on the dichlorbenzoic acids indicates the presence of more than one type of pyrocatechase.

3.5 Discussion

Microorganisms able to degrade chlorobenzoic acids, are usually restricted with respect to the range of chlorobenzoic acid isomers which can be metabolized. In aerobic microorganisms, this limitation is often attributed to the narrow substrate specificity of the first enzyme in the pathway, benzoate 1,2-dioxygenase (Reineke and Knackmuss 1978a). *A. denitrificans*, strains BRI 3010 and BRI 6011 utilized most of the 2-chloro substituted isomers of dichlorobenzoic acids. The only 2-chloro substituted dichlorobenzoic acid not utilized by BRI 6011 was 2,6-DCBA. The fact that 2,6-DCBA entered the cells and induced pyrocatechase (Table 3.3), but was not metabolized by BRI 6011, suggests that carbon six of the aromatic ring must remain unsubstituted if metabolization is to occur. Examination of the properties of the benzoic acid dioxygenase should help clarify this. Increased rates of chlorinated substrate utilization were observed in the presence of the alternate carbon source, yeast extract. This also occurs in the metabolism of pentachlorophenol (PCP) (Topp et al.1988), 2,4-D and 3,5-DCBA (Kim and Maier 1986). The alternate carbon source allowed the culture to rapidly increase its biomass and consequently the concentration of enzymes responsible for the metabolism of the recalcitrant substrate.

Resting cells demonstrated differential uptake of mono- and dichlorobenzoic acids. Highest rates of uptake occurred with 2-CBA, except when resting cells had been previously grown on 2,5-DCBA, in which case, 2,5-DCBA was the preferred substrate. A similar phenomenom was observed with oxygen uptake responses, where 2-CBA was oxidized at the highest rate and only upon induction with 2,5-DCBA did the rate of 2,5-DCBA oxidation approach that of 2-CBA (data not shown). The increased rate of 2,5-DCBA uptake, did not correspond to an increase in uptake for the other substrates. It is

possible that two different uptake systems with different affinities for 2,5-DCBA, may be present in 2,5-DCBA grown cells. One of the uptake systems would appear to be specific for 2,5-DCBA, the other less specific, and shared by the other chlorobenzoic acids tested. It is through the latter system that competition between 2,4-DCBA and 2,5-DCBA was observed. Information on the role of permeases in substrate specificity and uptake of haloaromatics is rather scarce, although two different permease systems for halobenzoates have been suggested based on concentration dependent oxidation and uptake rates (Taylor et al. 1979).

Degradation of chlorobenzoic acids by both strains proceeded by intradiol cleavage of the aromatic ring. In BRI 3010 and BRI 6011, 2-CBA induced pyrocatechase activity directed against catechol with essentially no activity against chlorocatechols. The specific activities obtained with 2-CBA were comparable to those obtained with benzoic acid and hydroxybenzoates, substrates not expected to induce pyrocatechase activity against chlorocatechols. The metabolizable dichlorobenzoic acids were good inducers of pyrocatechase activity directed against both catechol and chlorocatechols, with 2,4-DCBA being the most potent. The induction and expression of pyrocatechase activities in BRI 3010 and BRI 6011 appears to be similar to that reported for *Pseudomonas* (Dorn and Knackmuss 1978a). Pyrocatechase I is expressed during growth of *Pseudomonas* on benzoate, or chlorobenzoates, whereas pyrocatechase II (chlorocatechol 1,2-dioxygenase) is induced by growth on chlorobenzoates and shows high relative activites for chlorocatechols. In other cases, however, both isoenzymes are induced by growth on benzoate or chlorobenzoates (Hartmann et al. 1979). Although BRI 6011 grown on 2,4-DCBA expressed the highest pyrocatechase activity, the specific uptake rate for 2,4-DCBA was lower than for 2-CBA and 2,5-DCBA.

These observations suggest that the rate of chlorobenzoic acid utilization is mediated by at least two factors; the substrate uptake system, and an early enzyme in the degradation pathway, such as benzoic acid dioxygenase or pyrocatechase. Variation in pyrocatechase specific activities, dependent on the growth substrate, has been observed previously, (Hartmann et al. 1979), and demonstrates that knowledge of the regulatory systems is crucial in order to explain the induction of different pyrocatechase isoenzymes.

Two strains of *Alcaligenes denitrificans*, isolated from the same source and with virtually identical biochemical characteristics, differed in their ability to degrade two dichlorobenzoic acid isomers. The increased versatility towards chlorobenzoic acid metabolism by BRI 6011 may have been due to the broadening of the benzoate 1,2-dioxygenase specificity by either mutational events or by plasmid exchange and recombination. This and the role of substrate specific uptake systems in the acquisition of substrate degradation potential are being investigated.

Section 4

Purification and Properties of Chlorocatechol 1,2-Dioxygenase from *Alcaligenes denitrificans* BRI 6011

4.0 Preface

This section describes the the purification and characterization of chlorocatechol 1,2-dioxygenase from *Alcaligenes denitrificans* BRI 6011. Comparisons are made with the chlorocatechol 1,2-dioxygenase from *Pseudomonas sp.* B13, in order to ascertain the role this enzyme plays in the increased versatility for chlorobenzoic acid degradation of *A. denitrificans* over *Pseudomonas sp.* B13.

This section is drawn from the publication:

Miguez, C.B., Greer, C.W., and Ingram, J.M. 1993. Purification and properties of chlorocatechol 1,2-dioxygenase from *Alcaligenes denitrificans* BRI 6011. Can. J. Microbiol. **39**:1-5.

4.1 Abstract

The specific activity of chlorocatechol 1,2-dioxygenase from *Alcaligenes denitrificans* BRI 6011 was found to be maximal in the early logarithmic growth phase. The enzyme was purified from cultures at mid-log phase of growth using ammonium sulfate fractionation, Phenyl-Sepharose and DEAE-Sepharose chromatography. The protein gave a single band by SDS-PAGE with an apparent molecular weight of 33,000, and the temperature and pH optima were 30°C and 7.5, respectively. Catechol, 3-chlorocatechol (3-CC), 4-CC, 3,4-dichlorocatechol (3,4-DCC), 3,5-DCC, 3,6-DCC, 3-methylcatechol (3-MC) and 4-MC served as substrates for the enzyme. The V_{max} for the dichlorocatechols were similar, while those for the mono-chlorinated and methylated catechols were higher. The K_m for all the chlorinated catechols were typically below 1 μM, while those for catechol and the methylated catechols were above 10 μM.

Key words: Chlorocatechol 1,2-dioxygenase, *Alcaligenes denitrificans*, purification, characterization, chlorobenzoic acid degradation.

4.2 Introduction

A key enzyme in the degradation of aromatic compounds by aerobic microorganisms is catechol 1,2-dioxygenase, referred to as type I catechol dioxygenase, or pyrocatechase I (Dorn and Knackmuss 1978a). Several catechol 1,2-dioxygenase type enzymes have been described that have a higher affinity for chlorinated derivatives of catechol, and have been referred to as chlorocatechol 1,2-dioxygenase, type II catechol dioxygenase or pyrocatechase II (Dorn and Knackmuss 1978a, 1978b; Grishchenkov et al. 1983; Hartmann et al. 1979). These latter enzymes are important in the degradation of chlorinated aromatic compounds such as chlorobenzenes, chlorobenzoates, chlorophenols and the chlorophenoxyacetic acid herbicides, and the genes encoding them are typically located on large catabolic plasmids (Chatterjee et al. 1981; Chatterjee and Chakrabarty 1983; Ghosal et al. 1985; van der Meer et al. 1991a). Chlorocatechol 1,2-dioxygenase genes share a high degree of homology with each other (up to 75.7 %) in contrast to the low homology (22%) these enzymes share with the chromosomally encoded catechol 1,2-dioxygenase, as predicted by amino acid sequence (van der Meer et al. 1991b). Although the chlorocatechol 1,2-dioxygenases demonstrate strong evolutionary relatedness, they possess different affinities for chlorinated catechols.

Alcaligenes denitrificans BRI 6011 is distinctive in its ability to degrade a range of chlorobenzoic acid isomers including 2-chlorobenzoic acid (2-CBA), 2,3-dichlorobenzoic acid (2,3-DCBA), 2,4-DCBA and 2,5-DCBA (Miguez et al. 1990). Crude cell extracts prepared from *Alcaligenes denitrificans* BRI 6011 grown on 2,5-DCBA exhibit high relative activities with 3-chlorocatechol (3-CC) and 4-CC as compared with catechol, suggesting the involvement of chlorocatechol 1,2-dioxygenase. In addition to 3-CC, and 4-CC, 3,4-

dichlorocatechol (3,4-DCC), 3,5-DCC, 3,6-DCC, 3-methylcatechol (3-MC), and 4-MC can also serve as substrates. We have previously shown that the induction and expression of the chlorocatechol 1,2-dioxygenase activity in *Alcaligenes denitrificans* BRI 6011 appears to be similar to that of *Pseudomonas sp.* B13 (Miguez et al. 1990). However, *Pseudomonas sp.* B13 possesses a much narrower chlorobenzoic acid substrate specificity, capable of degrading only 3-CBA, an isomer not metabolized by BRI 6011. In order to ascertain the role which chlorocatechol 1,2-dioxygenase plays in the chlorobenzoic acid substrate versatility of *Alcaligenes denitrificans* BRI 6011, the enzyme was purified, characterized and compared to the chlorocatechol 1,2-dioxygenase from *Pseudomonas sp.* B13.

4.3 Material and Methods

Organism

The isolation and characterization of *Alcaligenes denitrificans* BRI 6011 have been previously described (Miguez et al. 1990).

Growth study

A. denitrificans BRI 6011 was grown in 2 litres of a minimal salts medium (MSM) (Miguez *et al.* 1990) containing 5 g/l yeast extract (Y.E./MSM) and 0.5 mM 2,4-DCBA in Erlenmeyer flasks at 30°C, and at 250 rpm. At the indicated sampling times, aliquots were removed for optical density and substrate concentration measurements as previously described (Miguez et al. 1990). Crude lysates of cells representative of the different growth phases of the culture were prepared by sonication as previously described (Miguez *et al.* 1990).

Oxidation of chlorinated catechols

Oxygen uptake was measured at 30°C using a Clark-type oxygen electrode (model 5300, Yellow Springs Instrument Co., Yellow Springs, Ohio). A 3-ml reaction mixture contained lysate (300-400 µg protein/100 µl lysate) in phosphate buffer (10 mM. pH 7.0), and the reaction was initiated by injecting 30 µl of 10 mM substrate (chlorinated catechols) dissolved in methanol. Oxygen consumption rates were corrected for endogenous respiration.

Production of chlorocatechol 1,2-dioxygenase by Alcaligenes denitrificans BRI 6011

A. denitrificans was grown in 2,5-DCBA (1.0 mM) supplemented YE/MSM as described above. Cells were harvested in the mid-log phase of growth, centrifuged (10,000 x *g*, 20 min, 4°C), washed twice with sterile MSM, resuspended in 50 ml TE*ß* buffer (50 mM TRIS/HCl, 1 mM EDTA, 1 mM *ß*-mercaptoethanol, pH 8.0). This buffer was used throughout the purification of the enzyme. Approximately 10 g of wet cells were obtained from 8 L of medium. The washed cell suspension was disrupted in a French press (Aminco, Illinois, U.S.A.) at 14,000 psi. Cell debris was removed by centrifugation at 19,000 x *g* for 30 min yielding 45 ml of cell-free extract (crude extract). The supernatant was assayed for protein and pyrocatechase activity.

Chlorocatechol 1,2-dioxygenase purification

Ammonium sulfate fractionation

To the crude extract, solid ammonium sulfate was added to 45% saturation. The mixture was allowed to equilibrate by gentle stirring for 30 min at 4°C. The precipitate was removed by centrifugation (19,000 x *g* for 30 min). Solid ammonium sulfate was added to the supernatant to 65% saturation and stirred overnight at 4°C. The precipitate was collected by centrifugation and resuspended in 20 ml of TE*ß* in 2M KCl. (Ammonium sulfate extract).

Chromatography on Phenyl-Sepharose CL-4B

The ammonium sulfate extract was applied to a Phenyl-Sepharose column (1.8 X 20 cm), equilibrated with TE*ß* in 2M KCl. The enzyme was eluted with a linear gradient of decreasing KCl. The flow rate was 0.5 ml/min. One-ml fractions were collected, and the active fractions were equilibrated by dialysis with TE*ß*, the starting buffer for the DEAE-Sepharose chromatography.

Chromatography on DEAE-Sepharose CL-6B

The dialysed solution was applied to a DEAE-Sepharose CL-6B column (1.8 X 20 cm) previously equlibrated with TEβ. The enzyme was eluted with a linear gradient of increasing NaCl to 0.5M. The flow rate was 0.5 ml/min, and 1 ml fractions were collected. The active fractions were concentrated and desalted by ultrafiltration (Amicon) using a YM-10 membrane.

Pyrocatechase assay

Assay of chlorocatechol 1,2-dioxygenase activity was performed spectrophotometrically by monitoring the rate of formation of the ring cleavage product, *cis,cis*-muconic acid or its substituted derivatives, as previously described (Miguez *et al.* 1990).

Kinetic characterization of chlorocatechol 1,2-dioxygenase

The Michaelis-Menten constant was determined with catechol, and 3-MC concentrations ranging from 1.0 to 400 μM. For the chlorinated catechols, the concentrations ranged from 0.05 to 10 μM. The enzyme assays were done in duplicate as described above. The apparent K_m and V_{max} for each of the substrates were determined from the linearized forms of the Michaelis-Menten equation; Lineweaver-Burk and Eadie-Hofstee plots (Fersht 1977).

Determination of pH profile

The effects of pH on enzyme activity were performed as described above with the following buffer systems; sodium acetate (50 mM) pH 5-6; potassium phosphate (50 mM) pH 6-7; Tris-HCl (50 mM) pH 7-9 and glycine-sodium hydroxide (50 mM) pH 9-10. Substrates for the enzyme assays were catechol and 3-CC. Enzyme was pre-incubated at the corresponding pH for 1 min at

30°C prior to the assay. In order to determine enzyme stability at these pH values, the enzyme was exposed in an incubation mixture containing Tris/acetate (50 mM) buffer and β-mercaptoethanol (1 mM) at various pH for 5 min. Enzyme activity was then assayed in the same buffer adjusted to pH 8.

Determination of temperature profile

The effect of temperature on enzyme stability was tested by exposure to 10-60°C in TEβ buffer for a duration of 60 min. The effect of long term exposure at low temperatures was carried out at 4°C and -20°C. The enzyme assay conditions are described above. Substrates for the enzyme assays were catechol, 3-CC, and 3,6-DCC.

Effect of heavy metals on enzyme activity.

The effect of Cu^{2+}, Ag^{2+}, and Hg^{2+} cations on enzyme activity was assayed. The enzyme (10 µg protein) was incubated at 4°C for 24 h in the following buffered mixture: Tris/HCl (20 mM); and either $CuCl_2$ (0.1 mM), $AgCl_2$ (0.1 mM), or $HgCl_2$ (0.1 mM). β-mercaptoethanol (1 mM) was added to the reaction mixture prior to the introduction of substrate. The final pH was 8.0. The reaction was started by the addition of catechol or substituted catechol (40 µl from a 10 mM stock solution in distilled water) to 960 µl of the incubation mixture. Enzyme activity was assayed spectrophotometrically as described above.

Polyacrylamide Gel Electrophoresis

Enzyme purity and apparent molecular weight determination were by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) in 11-23% gradient gels (PhorCast, Amersham, U.K.) using the high range M_r

(14,300-200,000) protein standards (Rainbow Markers, Amersham, U.K.). Between 20 and 50 µg of protein was layered on the gel surface. The gels were run at a constant current of 25 mAmp until the dye reached the gel bottom. The protein was stained with Coomassie blue R250. The apparent M_r was calculated from a standard linear regression curve of reference proteins.

Analytical

2,4-DCBA was determined by HPLC as previously described (Miguez et al. 1990). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

4.4 Results

Growth phase vs oxygen uptake with key metabolites

Growth of *A. denitrificans* BRI 6011 on chlorinated benzoic acids induces the synthesis of chlorocatechol 1,2-dioxygenase. The corresponding chlorinated catechol is a key metabolite in the degradation scheme of substituted benzoic acids. The oxidation rate of this metabolite is a limiting step in the overall degradation. Maximal specific oxygen uptake with 3,5-DCC as substrate was obtained with cell extracts originating from the early logarithmic growth phase (Fig.4.1). A similar result was obtained with 4-CC as substrate.

Chlorocatechol 1,2-dioxygenase purification

The purification of the enzyme is summarized in Table 4.1. This procedure resulted in a 13% yield and a 42-fold increase in the specific activity of the enzyme. A significant amount of the enzyme activity was lost in the Phenyl-Sepharose purification step. The protein peak containing the chlorocatechol 1,2-dioxygenase activity was rather broad and was eluted between 1.3-2.0 M KCl. Consequently, only fractions with high specific activity were retained for subsequent purification on DEAE-Sepharose. The enzyme was eluted between 0.1-0.16 M NaCl. The peak of enzyme activity was eluted at 0.13 M NaCl. Using polyacrylamide gradient gel electrophoresis, the desalted and concentrated enzyme was analyzed. The resultant polypeptide from the purified enzyme migrated as a single band with an apparent M_r of 33,000.

Figure 4.1

Effect of the phase of growth of the culture growing in 0.5 mM 2,4DCBA(O) supplemented with 5 g/l yeast extract on the specific oxidation rate of 3,5-DCC (▲). Growth was monitored by optical density at 660 nm (O.D. 660 nm)(●). Utilization of 2,4-DCBA was determined by HPLC. Assay conditions for oxygen consumption rates are descibed in Materials and Methods.

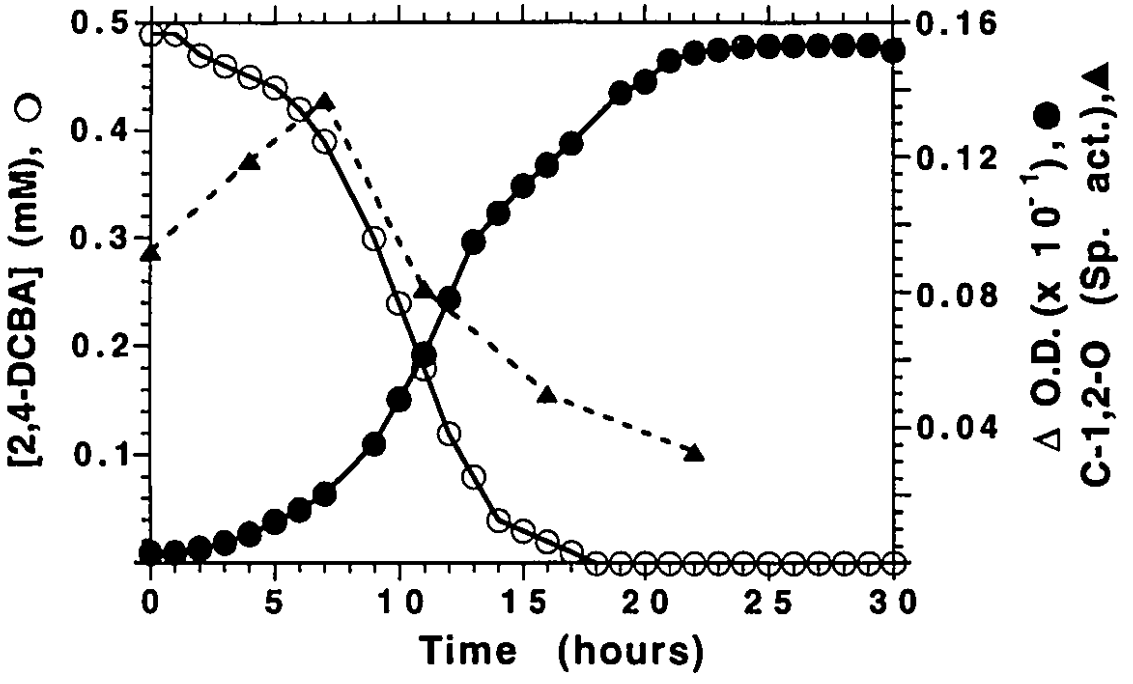

0.5
0.4
0.3
0.2
0.1
[2,4-DCBA] (mM), ○
0.16
0.12
0.08
0.04
Δ O.D. (x 10⁻¹), ●
C-1,2-O (Sp. act.),▲
0
5
10
15
20
25
30
Time (hours)

Table 4.1 Summary of the purification of chlorocatechol 1,2-dioxygenase from *A. denitrificans* BRI 6011

Purification steps	Total volume (ml)	Total activity* (units)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude extract	45	364.6	6159	59.2	100	1
Ammonium sulphate fractionation	40	327.3	1996	164.0	90	3
Phenyl-Sepharose	145	96.0	114	841.9	26	14
DEAE-Sepharose	24	47.8	19	2512.9	13	42

*One unit of activity is defined as that amount which catalyzes the formation of 1 μmol of product per minute.

Kinetic characterization of chlorocatechol 1,2-dioxygenase

The apparent Michaelis-Menten constant (K_m) and maximum velocities (V_{max}) for catechol and substituted catechols (Table 4.2) were obtained graphically from Eadie-Hofstee plots (Fig.4.2 to Fig.4.7). The chlorocatechol 1,2-dioxygenase from BRI 6011 has a high affinity for chlorinated catechols, in particular 3,5-DCC, and 3,6-DCC. The highest V_{max} and K_m were obtained with 3-MC as substrate.

Physico-chemical properties

The effect of temperature on enzyme activity was assayed from 10 to 60°C. Maximal activity was observed at 40°C, but optimal activity and stability was observed at 30°C, and the three test substrates, catechol, 3-CC and 3,6-DCC, behaved identically (Fig.4.8). Increasing the temperature to 40°C, 50°C, and 60°C resulted in 42%, 76%, and 95% inactivation, respectively. Storage of the enzyme in TEβ at 4°C and -20°C for one week resulted in a 10% and 60% loss of activity, respectively.

The pH maximum for enzyme activity with catechol as substrate was observed at pH 7.5 (Fig.4.9). A similar profile was observed with 3-CC as the substrate. Activity decreased quite rapidly below and above this pH and 20% and 10% activity remained at pH 5.0 and 10.0, respectively. When the enzyme was incubated for 5 min at different pH values, irreversible inactivation occurred below pH 6.5. Full activity of the enzyme exposed to pH 7.5-10 was recovered when assayed at pH 7.5 in the same buffer.

The enzyme exhibited different sensitivities to the heavy metals assayed. The presence of Cu^{2+}, Hg^{2+}, and Ag^{2+} cations resulted in 97%, 79%, and 52% inhibition of enzyme activity, respectively. The buffer utilized in these assays did not contain EDTA (1 mM), since partial alleviation of inhibition was observed in its presence.

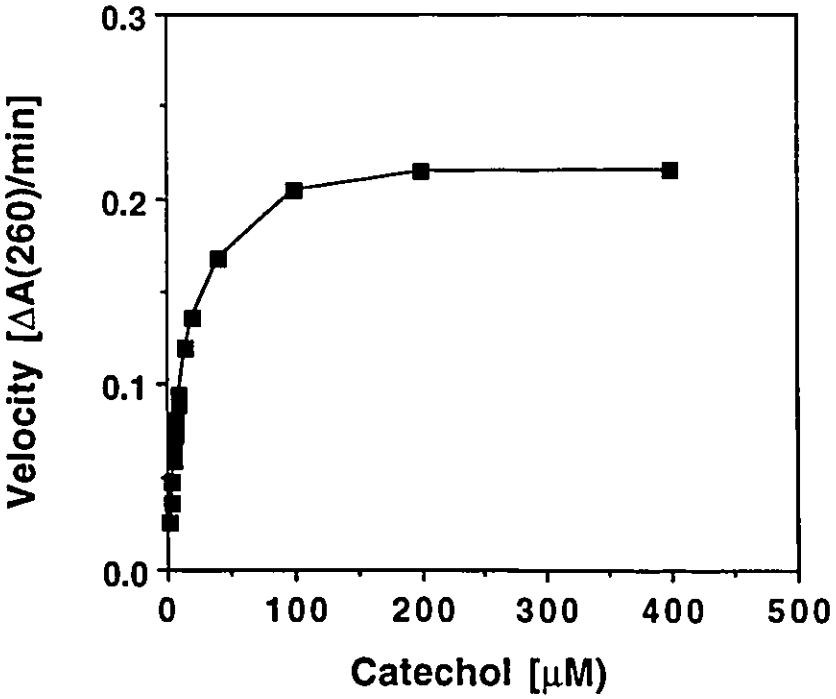
Table 4.2 Apparent *Km* and *Vmax* values of substituted catechols for chlorocatechol 1,2-dioxygenase from *A. denitrificans* (BRI 6011)

Substrate	*Vmax* (ΔA_{260}/min)	*Km* (μM)
catechol	0.235	15.31
3-methylcatechol	0.454	21.06
3-chlorocatechol	0.293	0.59
4-chlorocatechol	0.217	0.44
3,5-dichlorocatechol	0.130	0.34
3,6-dichlorocatechol	0.127	0.39

Figure 4.2

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with catechol concentrations ranging from 1.0-400 μM.

Michaelis-Menten



Eadie-Hofstee

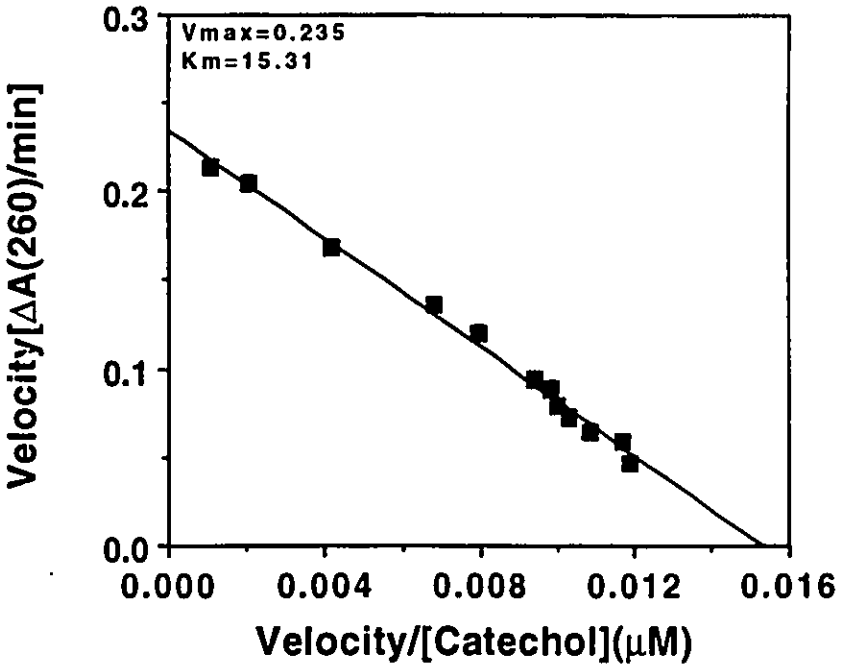
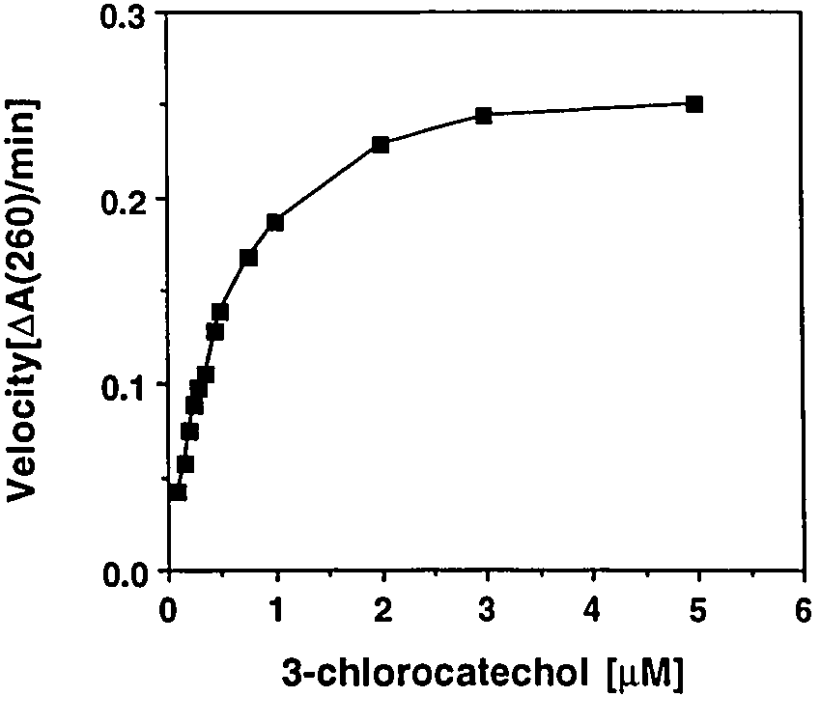


Figure 4.3

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with 3-chlorocatechol concentrations ranging from 0.05-5.0 μM.

Michaelis-Menten



Eadie-Hofstee

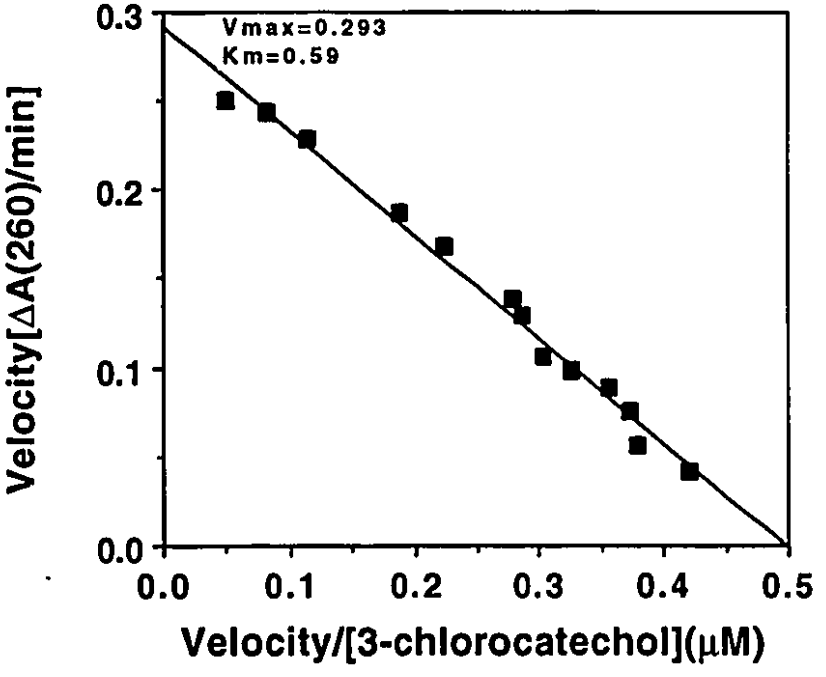
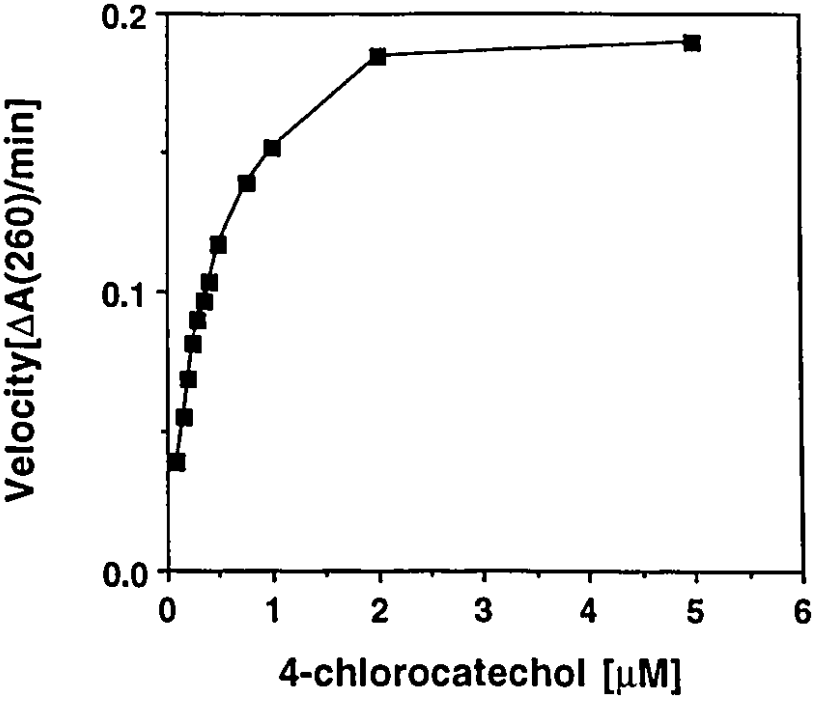


Figure 4.4

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with 4-chlorocatechol concentrations ranging from 0.05-5.0 μM.

Michaelis-Menten



Eadie-Hofstee

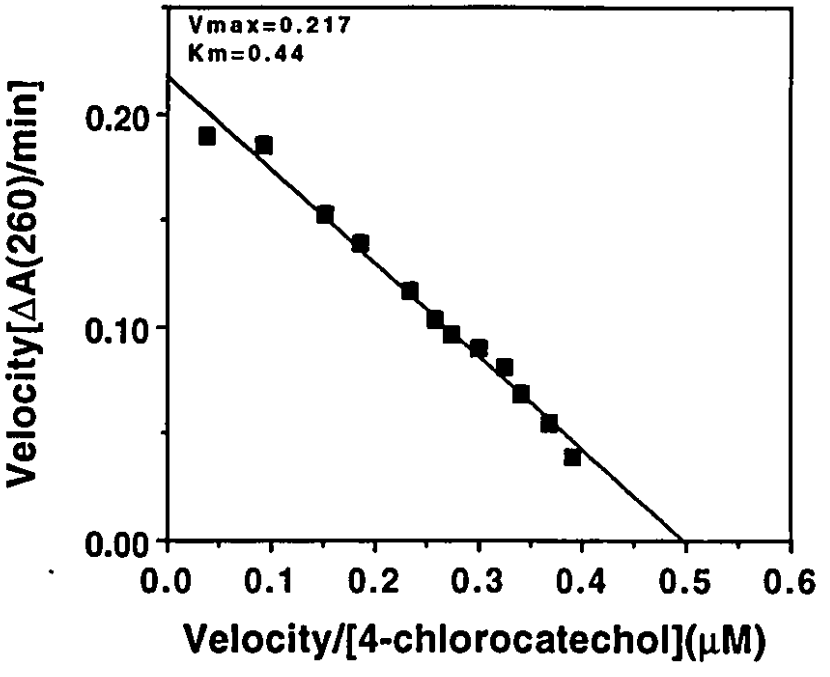
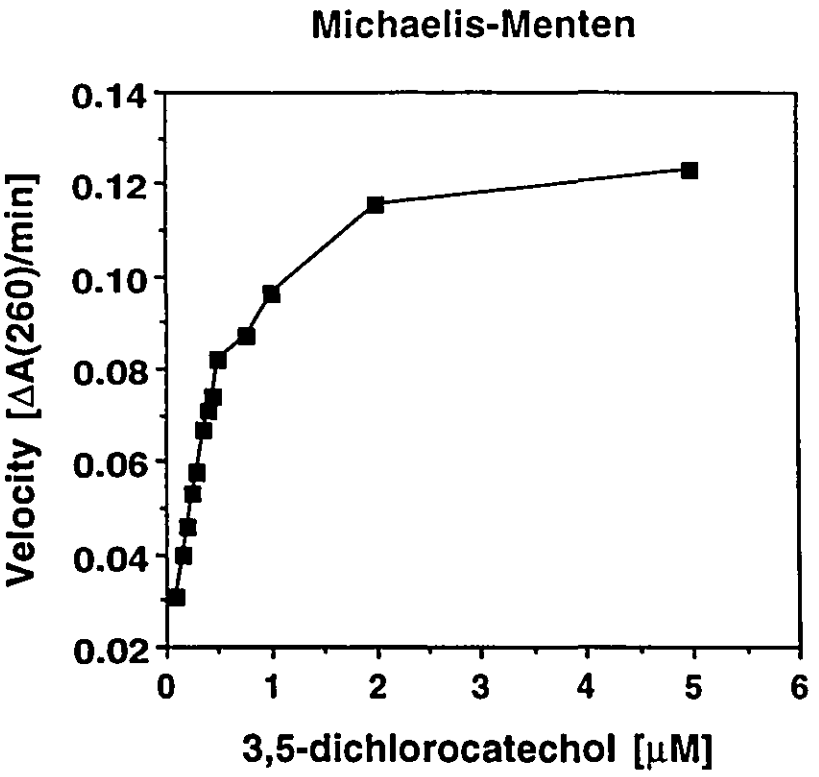


Figure 4.5

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with 3,5-dichlorocatechol concentrations ranging from 0.05-5.0 μM.


Michaelis-Menten
Velocity [ΔA(260)/min]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
3,5-dichlorocatechol [μM]

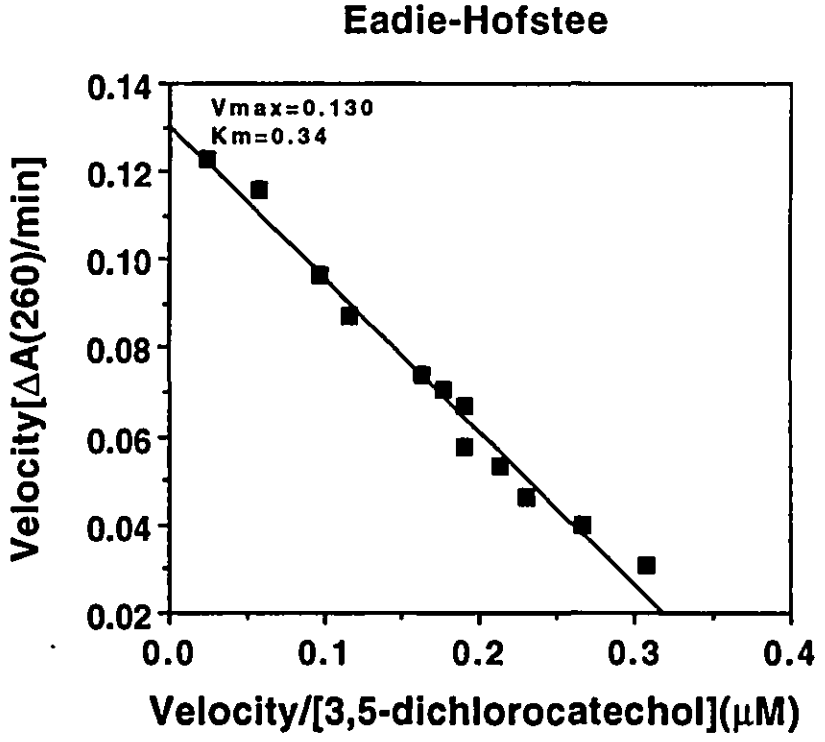
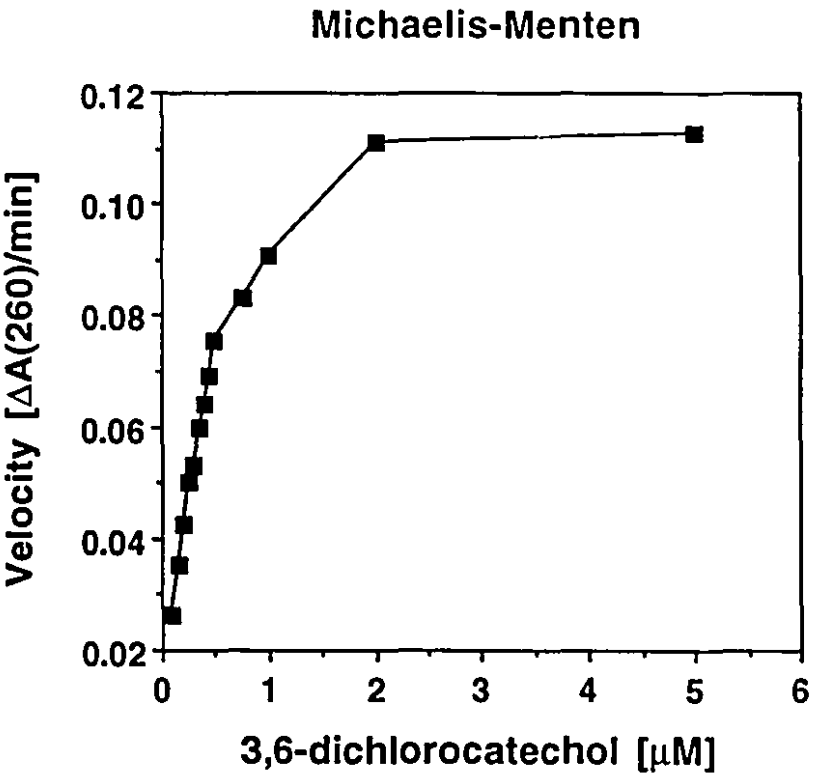

Eadie-Hofstee
Vmax=0.130
Km=0.34
Velocity[ΔA(260)/min]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
0.1
0.2
0.3
0.4
Velocity/[3,5-dichlorocatechol](μM)

Figure 4.6

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with 3,6-dichlorocatechol concentrations ranging from 0.05-5.0 μM.


Michaelis-Menten
Velocity [ΔA(260)/min]
0.12
0.10
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
3,6-dichlorocatechol [μM]

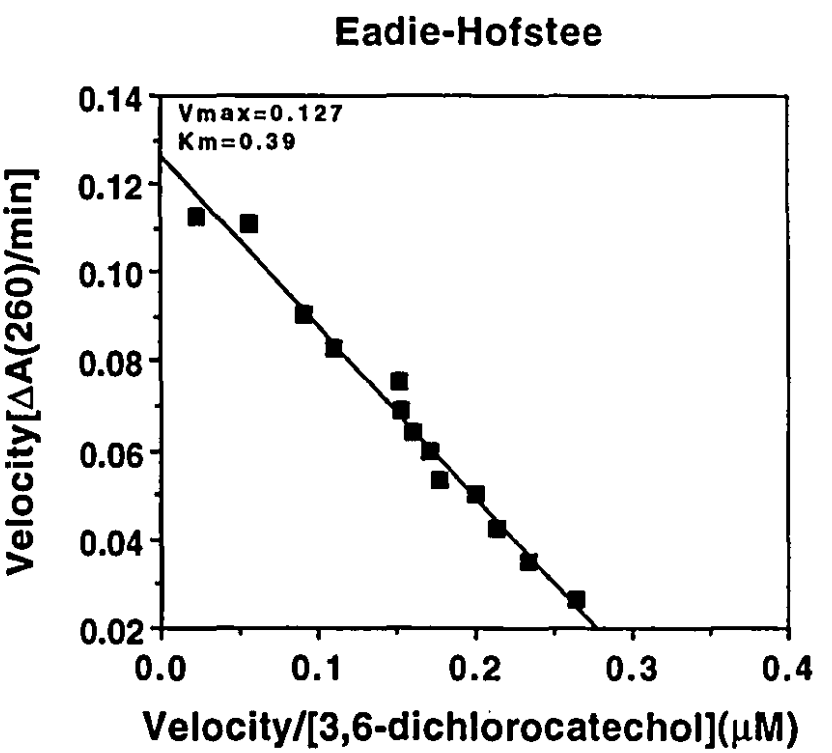
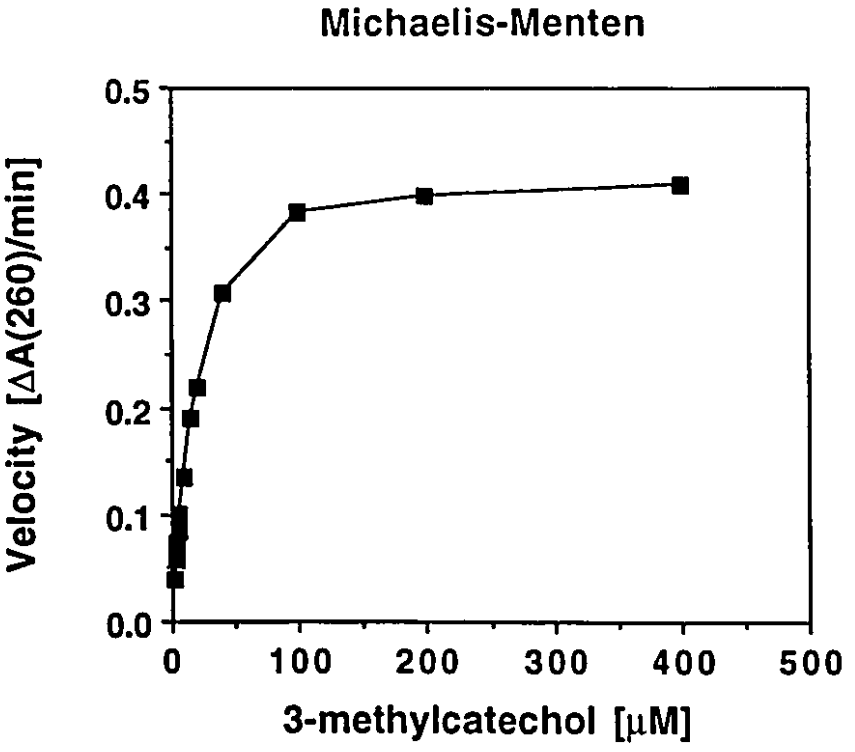

Eadie-Hofstee
Vmax=0.127
Km=0.39
Velocity[ΔA(260)/min]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
0.1
0.2
0.3
0.4
Velocity/[3,6-dichlorocatechol](μM)

Figure 4.7

Michaelis-Menten and Eadie-Hofstee plots for chlorocatechol 1,2-dioxygenase activity determined with 3-methylcatechol concentrations ranging from 1.0-400 μM.


Michaelis-Menten
Velocity [ΔA(260)/min]
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
3-methylcatechol [μM]

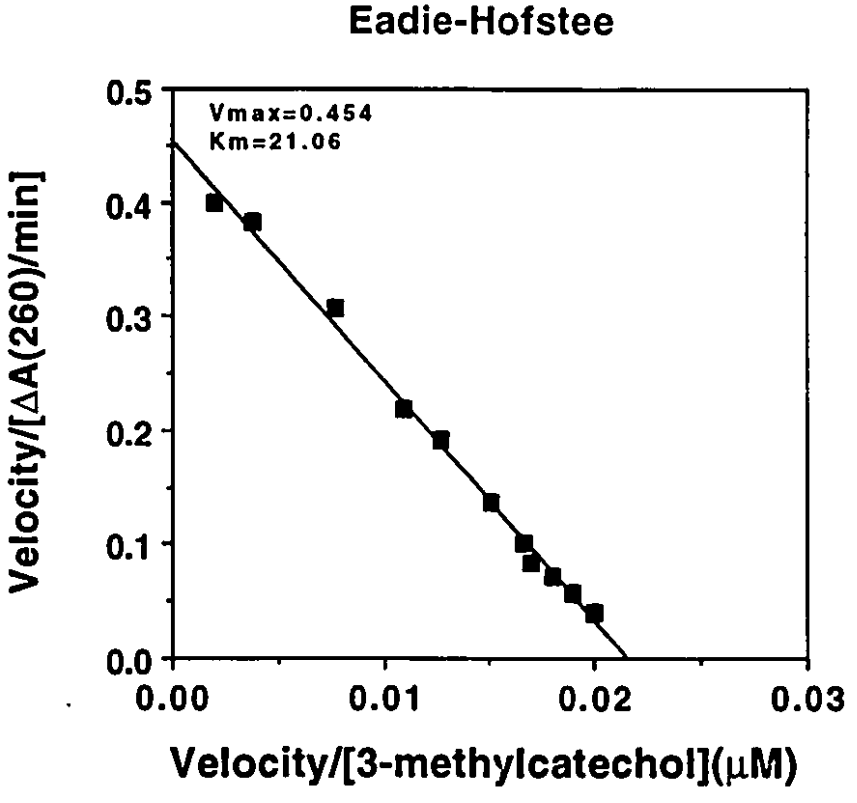

Eadie-Hofstee
Vmax=0.454
Km=21.06
Velocity/[ΔA(260)/min]
0.5
0.4
0.3
0.2
0.1
0.0
0.00
0.01
0.02
0.03
Velocity/[3-methylcatechol](μM)

Figure 4.8

Effect of temperature on chlorocatechol 1,2-dioxygenase activity. The enzyme was incubated in TEß buffer at the indicated temperatures for a duration of 1 minute. Catechol(□), 3-CC(▲) and 3,6-DCC(○) served as substrates. For assay conditions see Materials and Methods.

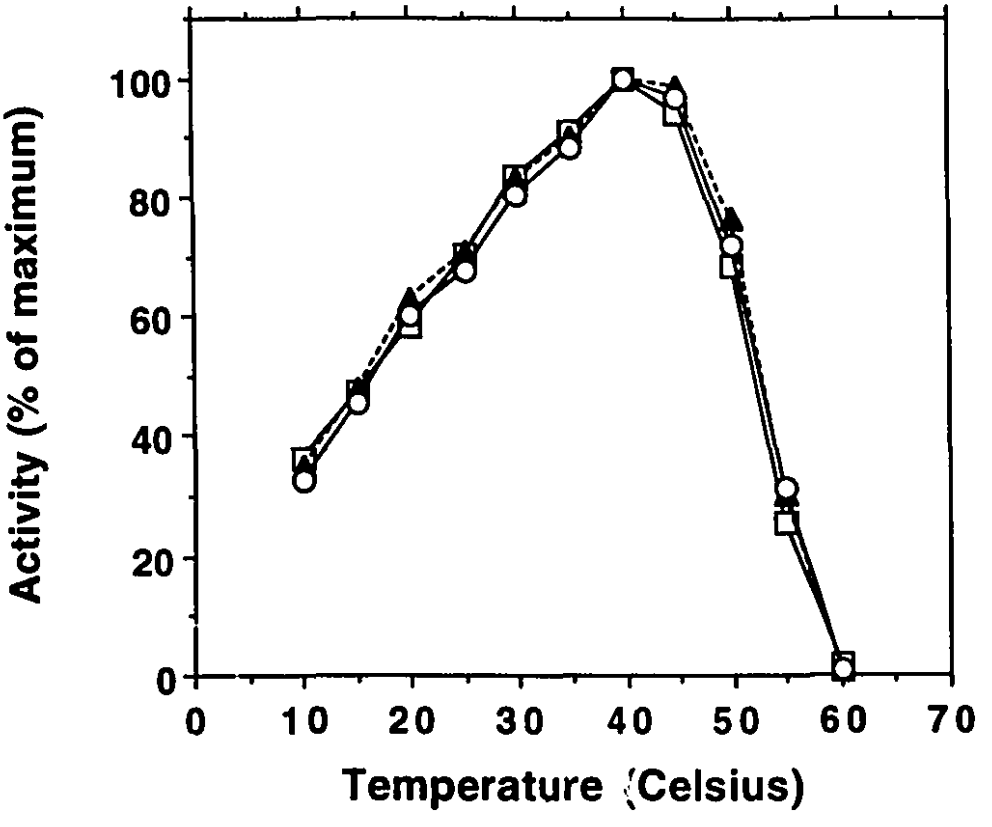
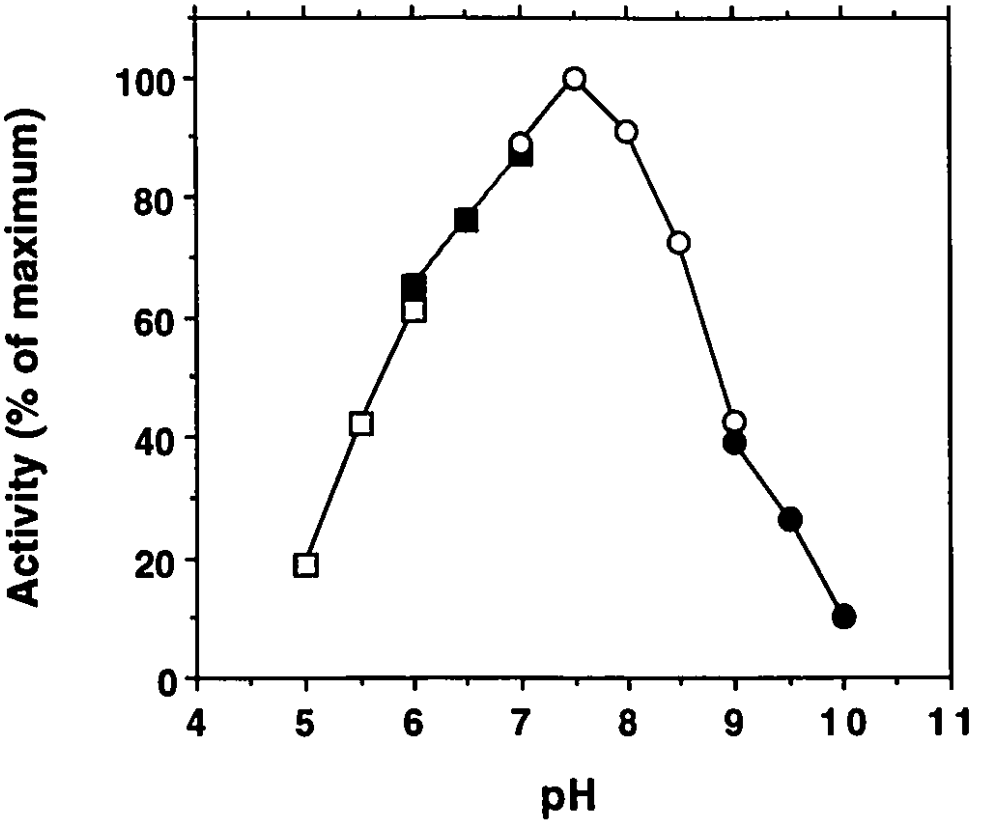

Activity (% of maximum)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (Celsius)

Figure 4.9

Effect of pH on chlorocatechol 1,2-dioxygenase activity. The following buffers, all at 50 mM were used for the different pH ranges indicated: sodium acetate, pH 5-6, (❑); potassium phosphate, pH 6-7, (■); Tris-HCl, pH 7-9 (○); and glycine-sodium hydroxide pH 9-10 (●). For assay conditions see Materials and Methods.


Activity (% of maximum)
100
80
60
40
20
0
4
5
6
7
8
9
10
11
pH

4.5 Discussion

Microorganisms capable of metabolizing cholorobenzoic acids are essential for the complete mineralization of recalcitrant compounds such as polychlorinated biphenyls (PCBs) and certain chloro-organic herbicides. *Alcaligenes denitrificans* BRI 6011 is well adapted for the degradation of chlorobenzoic acids. The chlorocatechol 1,2-dioxygenase purified from this organism has low K_m values, typically below 1 μM, for 3-CC, 4-CC, 3,5-DCC, and 3,6-DCC. With the exception of 4-CC, these are all potential metabolites of the chlorobenzoic acids the organism is capable of utilizing.

The kinetic and physico-chemical characteristics, including temperature and pH optima and K_m profile, of chlorocatechol 1,2-dioxygenase from *A. denitrificans* BRI 6011 are similar to those of the chlorocatechol 1,2-dioxygenase from *Pseudomonas sp.* B13, the only other enzyme that has been purified and biochemically characterized (Dorn and Knackmuss 1978a, 1978b). Furthermore, the apparent molecular weights of both enzymes are identical (Ghosal and You 1988).

Several chlorocatechol 1,2-dioxygenase genes have been cloned and sequence analysis reveals that the genes from biodegradation pathways for chlorobenzoates, chlorobenzenes and chlorophenoxyacetates are relatively homologous (Frantz and Chakrabarty 1987, Ghosal and You 1988, 1989, van der Meer et al. 1991b). Crude enzyme preparations of native and cloned chlorocatechol 1,2-dioxygenases have been compared, and although specific activities against various chlorinated catechols were similar, the position and number of chlorines had a pronounced effect on substrate preference (van der Meer et al. 1991b). The chlorocatechol 1,2-dioxygenase from *A. denitrificans* demonstrated the highest activity against 3-CC, and cleaved both the dichlorinated catechol substrates tested (3,5-DCC and 3,6-DCC) at

approximately half that rate. This activity most closely approximates the activity of the chlorocatechol 1,2-dioxygenase encoded by the *clc*A gene from the pAC27 plasmid of *P. putida*, which also shows a preference for 3-CC, but cleaves 3,6-DCC at half the rate of 3,5-DCC (van der Meer et al. 1991b). The nucleotide sequence of the gene encoding chlorocatechol 1,2-dioxygenase in *A. denitrificans* (Greer et al. 1991) is almost identical to the sequence reported for the *clc*A gene from *P. putida* (Ghosal and You 1988). The slight difference detected (3 out of 260 amino acids) may explain the variation in substrate preference demonstrated by these two enzymes.

The chlorocatechol 1,2-dioxygenases from *A. denitrificans*, *Pseudomonas* sp. B13 and *P. putida* (pAC27) appear to be virtually identical on the basis of biochemical and genetic studies. This is significant considering that these three bacteria were isolated from geographically distinct areas. *Pseudomonas sp.* B13 (Dorn et al. 1974) and *P. putida* (Chatterjee et al. 1981) were isolated from sewage samples in Germany and in the U.S., respectively, whereas *A. denitrificans* was isolated from soil samples from Smith Falls, Ontario, Canada, contaminated with Aroclor 1254 (Miguez et al. 1990). The chlorocatechol 1,2-dioxygenases from all three of these bacteria possess sufficient broad substrate specificity to accommodate a wide range of chlorinated catechols, hence the increased versatility for chlorobenzoic acid degradation of *A. denitrificans* over *Pseudomonas sp.* B13 can not be attributed to a more specialized chlorocatechol 1,2-dioxygenase. Rather, this versatility may be due to restrictions imposed by the substrate uptake systems and/or differences in the properties of the respective benzoate 1,2-dioxygenases, both of which are being investigated.

Section 5

Uptake of Benzoic acid and Chlorobenzoic acids

5.0 Preface

This section describes the uptake mechanisms for benzoic acid and chlorinated benzoic acids by *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011. The induction and expression of chlorocatechol 1,2-dioxygenase activity in *Alcaligenes denitrificans* strains BRI 3011 and BRI 6011 appear to be similar to that of *Pseudomonas sp.* B13 (Miguez et al. 1990). The purification and characterization of the chlorocatechol 1,2-dioxygenase from BRI 6011 has been described (Miguez et al. 1993), and on the basis of biochemical and genetic analyses, this enzyme is very similar to the chlorocatechol 1,2-dioxygenase from *Pseudomonas sp.* B13. This is an enzyme of crucial importance for the degradation of chlorobenzoic acids by all three microorganisms. However, these microorganisms differed in their ability to utilize different isomeric forms of chlorobenzoic acid. This difference in chlorobenzoic acid substrate versatility can therefore not be attributed to the activity of the chlorocatechol 1,2-dioxygenase enzyme. Uptake is generally considered to be the first step in the degradation pathway of most substrates. However, little is known concerning the uptake mechanisms of aromatic and haloaromatic compounds. For this reason it was decided to study the uptake of benzoic acid and 2,4-DCBA by BRI 3010, BRI 6011, and *Pseudomonas sp.* B13; and hopefully gain some insight into the role uptake plays in the chlorobenzoic acid substrate specificity of these microorganisms.

The work described in this section was done in Dr. MacLeod's laboratory (Macdonald College of McGill University) and under his supervision in collaboration with my co-supervisors, Drs. Greer and Ingram.

5.1 Abstract

The nature of benzoic and 2,4-dichlorobenzoic acid (2,4-DCBA) uptake by *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011, and by *Pseudomonas* sp. B13 was investigated. In all three organisms uptake of benzoic acid was inducible. For benzoic acid uptake into BRI 3010 monophasic saturation kinetics were obtained with apparent K_m and V_{max} values of 1.4 μM and 3.2 nmol/min/mg cell dry wt, respectively. In BRI 6011, biphasic saturation kinetics were observed suggesting the presence of two uptake systems for benzoic acid with distinct K_m (0.72 μM and 5.3 μM) and V_{max} values (3.3 nmol/min/mg cell dry wt and 4.6 nmol/min/mg cell dry wt), respectively. BRI 3010 and BRI 6011 accumulated benzoic acid against a concentration gradient by a factor of 8 and 10, respectively. A wide range of structural analogues, at 50-fold excess concentrations, inhibited benzoic acid uptake by BRI 3010 and BRI 6011, whereas with B13, only 3-chlorobenzoic acid (3-CBA) was an effective inhibitor. For BRI 3010 and BRI 6011, the inhibition by the structural analogues was not of a competitive nature. Uptake of benzoic acid by all three organisms was inhibited by KCN, the protonophore TCS, but to only a limited extent by arsenate, thus implicating the proton motive force (Δp) as the driving force for benzoic acid uptake. The ionophores, valinomycin and nigericin, were not effective inhibitors of benzoic acid uptake and consequently the component of the Δp responsible for driving uptake is not known at this time. Uptake of 2,4-DCBA by BRI 6011 was constitutive and saturation uptake kinetics were not observed. Uptake of 2,4-DCBA was inhibited by KCN, TCS, and partially by arsenate, but the compound was not accumulated intracellularly against a concentration gradient. Uptake of 2,4-DCBA by BRI 6011 appears to occur by passive diffusion into the cell down its concentration gradient with the downhill gradient being maintained by the metabolism of the compound by the cell.

5.2 Introduction:

Microbial metabolism of polychlorinated biphenyls (PCBs) by pure cultures of PCB degrading micro-organisms is generally incomplete and results in the formation of isomeric mixtures of chlorinated benzoic acids (CBAs) as dead end metabolites (Ahmed and Focht 1973; Furukawa et al. 1979; Masse et al. 1984). More complete degradation of PCBs by co-cultures consisting of both PCB and CBA degrading micro-organisms has been reported in which case, the rate of CBA removal from the growth medium dictated the rate of PCB degradation by the cells (Sylvestre et al. 1985; Sondossi et al.1992; Adriaens et al. 1989; Pettigrew et al. 1990; Shileds et al. 1985; Kohler et al. 1988).

Organisms capable of degrading 2-CBA (Taylor et al. 1979; Zaitsev and Karaservich 1984; Baggi 1985; Sylvestre et al. 1989; Engesser and Schulte 1989; Fetzner et al 1989a, 1989b), 3-CBA (Chatterjee et al. 1981; Dorn et al. 1974; Horvath and Alexander 1970; Haller and Finn 1979; Reber and Thierbach 1980) and 4-CBA (Keil et al. 1981; Klages and Lingens 1979; Marks et al. 1984a, 1984b; van den Tweel et al. 1987) have been isolated. Less, however, is known about micro-organisms with dichlorobenzoic acid degradative capabilities (Hickey and Focht 1990; Hernanadez et al. 1991). Two strains of *Alcaligenes denitrificans*, BRI 3010 and BRI 6011 have been isolated which can metabolize benzoic acid, 2-CBA, 2,3-dichlorobenzoic acid (2,3-DCBA) and 2,5-DCBA. In addition, strain BRI 6011 can also degrade 2,4-DCBA (Miguez et al. 1990). Degradation of benzoic acid and the CBAs by both organisms has been shown to proceed via intradiol cleavage of the aromatic ring. *Pseudomonas* sp. B13 also degrades benzoic acid via intradiol cleavage of the aromatic ring but is capable of degrading only 3-CBA (Dorn et al. 1974). The influence that uptake mechanisms have on substrate specificity in different microorganisms which degrade CBAs is not known.

Aromatic acids being lipophilic weak acids are generally assumed to traverse cell membranes by simple diffusion. Indeed, such compounds have been used for measuring pH gradients across membranes (Kihara and MacNab 1981: Kashket 1985). Periplasmic and binding proteins have not been implicated in the uptake or metabolism of aromatic compounds. Although there is extensive information on the degradative pathways of certain haloaromatic compounds, little is known concerning the mechanism(s) by which they are taken up by the cells. Several reports, however, have documented the existence of highly specific permeases in bacteria for the transport of aromatic acids including benzoic acid (Thayer and Wheelis 1982), 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (Wong et al. 1991), and 4-chlorobenzoic acid (Groenewegen et al. 1990).

In this study, the characteristics of the uptake processes for benzoic acid into *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011 and for 2,4-DCBA into BRI 6011, were examined. These and associated findings have provided insights into the possible role that uptake plays in dictating substrate specificity amongst BRI 3010, BRI 6011 and *Pseudomonas* sp. B13.

5.3 Materials and Methods

Organisms

The isolation and characterization of *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011 have been previously described (Miguez et al. 1990). *Pseudomonas* sp. B13 was isolated and characterized by Dorn et al. (1974).

Growth conditions

The cells were grown in minimal salts medium (MSM) (Miguez et al. 1990) supplemented with yeast extract (Y.E.) (5 g/l) and 5 mM BA, 1 mM 2,4-DCBA or 1 mM 2,5-DCBA. Cells were grown in Erlenmeyer flasks on a rotary shaker at 30°C.

Preparation of cells

Cells grown to late-log phase were harvested by centrifugation (7700 x g, 10 min, 4°C), washed three times with volumes of 50 mM potassium phosphate (pH 7.2) equal to the volume of the original medium. The washed pellet was resuspended in the same buffer to yield a final concentrated stock suspension of 7 mg cell dry weight/ml and kept on ice for the duration of the experiment.

Measurement of transport

Transport experiments were conducted using the Millipore filtration technique of Droniuk et al. (1987). Washed cells were added at a final density of 200 µg cell dry weight/ml to a reaction chamber containing phosphate buffer (pH 7.2) and chloramphenicol at a final concentration of 50 mM and 150 µg/ml, respectively. The reaction mixture was kept homogeneous and aerated by magnetic stirring while the temperature was maintained at 30°C by circulating water. The cell suspensions were allowed to equilibrate for 15 minutes before the addition of ^{14}C-labeled substrates. The reaction was started by adding [^{14}C]-labeled benzoic acid (ring-UL-labeled, 15 mCi/mmol) or [^{14}C]-labeled 2,4-DCBA (ring-UL-labeled, 54mCi/mmol) at a final concentration of 20 µM (5 µCi/µmol). The final incubation volume was 3.5 ml.

Uptake experiments under anaerobic conditions in the presence or absence of nitrate (KNO_3, final concentration of 60 mM) were performed essentially as the aerobic uptake experiments except that the incubation mixture was flushed continually with oxygen-free nitrogen gas. Cells were incubated for 30 minutes under these conditions prior to the addition of the labeled substrates.

After the addition of the labeled substrate at time zero, 0.5 ml samples were removed from the reaction mixture at regular time intervals. The samples were filtered through membrane filters (Millipore type HA, 0.45 μm). The cells on the filters were washed immediately with 10 ml phosphate buffer, placed in pre-heated scintillation vials and then exposed to an infrared lamp to stop metabolic activity in the cells. For each experiment a reaction mixture with boiled cells, or live cells in the presence of the protonophore 3,3',4',5-tetrachlorosalicylanilide (TCS) (10 μM) which was predetermined to inhibit uptake, or the reaction mixture without cells, was filtered in the same manner to determine any non-specific binding of radioactivity to the cells and/or filters.

Measurement of radioactivity

A 7 ml volume of Universol (ICN, Biochemicals, Irvine,CA) was added to the cooled dried filters in the scintillation vials. Radioactivity was determined using a Beckman LS-7500 liquid scintillation counter (Beckman Instruments Inc., Irvine, Ca) (Droniuk et al. 1987).

Extraction of intracellular substrate pools after transport

The procedure used was based on that described by Droniuk et al. (1987). The uptake of benzoic acid was as described earlier, except that the volume of the reaction mixture was reduced to 1.4 ml. Sampling was done only

once at 4 minutes. After each sample was filtered and washed, the filter was transferred to a centrifuge tube containing 10 ml water, kept at 90°C in a water bath. The uptake procedure was performed six times and the filters were pooled. In this manner, six filters with a combined cell dry weight of 600 µg adhering to them, were collected. After the last filter was added, the tubes were kept at 90°C for 15 minutes, cooled and centrifuged (40,000 x g, 15 min., 4°C). A second 10 ml portion of water was added to the filters which were then held at 90°C for 15 minutes. After centrifugation, the second supernatant was pooled with the first. The pooled supernatant was acidified to pH 2 and extracted twice with equal volumes of ethyl acetate. The organic phase was separated and evaporated to dryness with a stream of nitrogen. The extracted residue was then dissolved in a known volume of ethyl acetate.

Analysis of cell extracts by thin- layer chromatography

A known volume (5-10 µl) of concentrated radioactive cell extract was spotted on a silica gel plate (Baker-Flex 1B2, J.T. Baker Chemical Co., Phillipsburg, N.J.). The plate was developed according to the method of Reiner (1972) using a solvent system of 4:6:1 benzene-methanol-acetic acid. After drying with warm air, the plate was autoradiographed against X-ray film (X-Omat AR, Kodak, Rochester, N.Y.) for 96 hours. Spots representing benzoic acid or 2,4-DCBA were identified by comparing their migration on the plate to that of authentic samples. The spot representing benzoic acid or 2,4-DCBA was scraped into a scintillation vial for measurement of radioactivity. The intracellular concentration of the unchanged metabolite was calculated assuming an intracellular fluid volume of 1.5 µl/mg cell dry weight. This value was based on an average of 1.46 +/- 0.125 µl/mg cell dry wt determined from

figures reported for 8 other Gram-negative rod forms of similar dimensions (MacLeod, personal communication).

Cell dry weight determinations

Cell dry weights of cell suspensions were determined turbidimetrically at 660 nm (microsample spectrophotometer; model 300-N; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). An $O.D._{660}$ of 1.0 (1 cm light path) was equivalent to 0.396, 0.411, and 0.530 mg (dry weight) of cells per ml of *Alcaligenes denitrificans* strain BRI 3010, BRI 6011, and *Pseudomonas* sp. B13, respectively. These values were determined by washing fractions of cell suspensions three times in distilled water followed by centrifugation. The cell pellets were dried to constant weight by lyophilization.

Chemicals

The ring-UL-^{14}C-labeled-benzoic acid, valinomycin, and nigericin were obtained from Sigma Chemical Co.(St. Louis, Mo). The ring-UL-^{14}C-labeled-2,4-DCBA was obtained from Amersham (Oakville, Ontario, Canada). Non-radioactive aromatic substrates were obtained from Aldrich Chemical Company Inc. (Milwaukee, Wis.). All other chemicals used were reagent grade.

5.4 Results

A: Effect of culture age on uptake rates of benzoic acid and 2,4-DCBA

Previous studies with BRI 6011 have shown that the age of the culture had a profound effect on the maximal specific activity of chlorocatechol 1,2-dioxygenase, a crucial enzyme involved in the degradation of chlorobenzoic acids. Maximal specific oxygen uptake with chlorinated catechol substrates was obtained with cell extracts originating from the early logarithmic growth

phase (Miguez et al. 1992). In the present study, an effect of culture age on benzoic acid uptake was observed. Initial rates of uptake of benzoic acid into BRI 3010 and BRI 6011 were found to be highest in cells harvested in the late logarithmic phase of growth, whereas in *Pseudomonas* B13 the uptake rate was unaffected, by the age of the culture (Table 5.1). In contrast, uptake of 2,4-DCBA by BRI 6011 was not affected by the age of the culture (results not shown).

B: Inducibility of benzoic acid and 2,4-DCBA uptake, and the effect of an alternate respirable carbon source on uptake

Cultures (BRI 3010, BRI 6011 and *Pseudomonas* sp. B 13) grown repeatedly on minimal salts yeast extract medium were assayed for the ability to take up ^{14}C-labeled benzoic acid. In BRI 6011, the rate of uptake was low unless the growth medium was supplemented with benzoic acid (Table 5.2). This indicates that uptake of benzoic acid is mediated by an inducible uptake system. Uptake of benzoic acid into BRI 3010 and *Pseudomonas* sp. B13 was also inducible (results not shown). Uptake of 2,4-DCBA appears not to be inducible in BRI 6011 (Table 5.2). Indeed, the highest rates of 2,4-DCBA uptake were observed in cells grown on minimal yeast extract medium. The initial rate of uptake progressively decreased in cells grown on increasingly higher concentrations of 2,4-DCBA. It is also evident that benzoic acid and 2,4-DCBA do not share a common carrier in the membrane since an increase in benzoic acid uptake by benzoic acid induced cells did not result in a corresponding increase in 2,4-DCBA uptake. A concentration of benzoic acid in the growth medium which induced a 10-fold increase in the rate of uptake of benzoic acid

Uptake of 2,5-dichloro[carboxyl-^{14}C]benzoic acid was also examined, by the cells actually reduced the rate of uptake of 2,4-DCBA. and the initial rates

Table 5.1. Effect of growth phase on initial rates of benzoic acid uptake by *A. denitrificans* strains BRI 3010 and BRI 6011 and *Pseudomonas* sp. B13

Organism*	Mid-log	Late-log	Stationary phase
	Rate of Uptake† (nmol/min/mg cell dry wt)		
BRI 3010	0.33 ± 0.12	2.02 ± 0.18	0.59 ± 0.11
BRI 6011	0.63 ± 0.14	2.20 ± 0.13	1.02 ± 0.20
Pseudomonas B13	2.39 ± 0.19	2.43 ± 0.15	2.41 ± 0.18

* Cells were grown on minimal salts yeast extract medium supplemented with 5 mM benzoic acid. *Pseudomonas* B13 gave similar results when cells were also grown on minimal salts medium to which 10 mM benzoic acid but no yeast extract was added.

† results represent the mean and average deviation of at least two determinations.

Table 5.2. Effect of benzoic acid (BA) and 2,4-dichlorobenzoic acid (2,4-DCBA) in the growth medium on the subsequent capacity of *A. denitrificans* BRI 6011 to transport the compounds into the cells

Supplement*	Benzoic acid	2,4-DCBA
	Rate of Uptake† (nmol/min/mg cell dry wt)	
0	0.26 ± 0.03	0.63 ± 0.03
BA (5 mM)	2.51 ± 0.12	0.28 ± 0.02
2,4-DCBA (1 mM)	0.20 ± 0.01	0.41 ± 0.04
2,4-DCBA (2 mM)	0.21 ± 0.01	0.23 ± 0.02
2,4-DCBA (5 mM)	0.20 ± 0.02	0.12 ± 0.01

* to the minimal salts yeast extract medium. Cells were grown to late log phase.

† mean and average deviation of at least 2 determinations.

were similar to the uptake of ring-UL-^{14}C-labeled 2,4-DCBA (results not shown). However, since decarboxylation of the substrate occurs early in the degradation pathway, loss of radioactivity via $^{14}CO_2$ would result in an underestimation of initial uptake rates. Consequently, we did not proceed with this substrate as a model compound.

The addition of the metabolizable substrate, potassium citrate, to cell suspensions at a concentration of 1 mM, 10 min prior to the addition of the labelled substrate resulted in a 30 to 40% increase in the rate of uptake of benzoic acid by BRI 3010, BRI 6011 and *Pseudomonas* sp.B13, and a 20% increase in the rate of uptake of 2,4-DCBA (results not shown).

C: Kinetics of uptake of benzoic acid and 2,4-DCBA

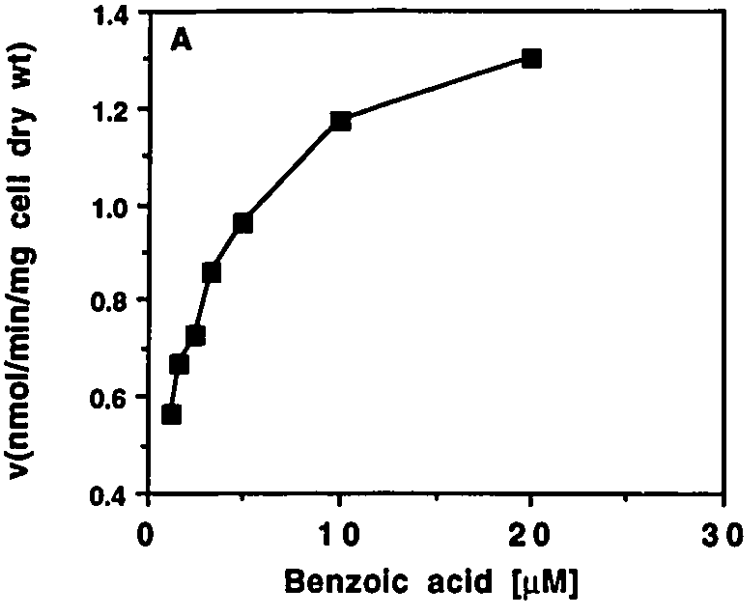
When the initial rate of uptake of benzoic acid by BRI 3010 was plotted against substrate concentration evidence for saturation kinetics was obtained (Fig. 5.1A). A double reciprocal plot of the data indicated monophasic saturation kinetics with an apparent affinity constant (K_m) of 1.4 μM and a maximal velocity (V_{max}) of 3.2 nmol/min/mg cell dry wt. (Fig. 5.1B).

Uptake of benzoic acid by BRI 6011 displayed biphasic saturation kinetics, (Fig. 5.2A) suggesting the presence of two saturable systems for benzoic acid uptake in this organism. When the reciprocals of the initial rates of uptake were plotted against the reciprocals of the benzoic acid concentration over the two concentration ranges, 0.1-1.67 μM and 5.0-20 μM, two K_m and V_{max} values were obtained (Figs. 5.2B and 5.2C). This provides evidence for a high and a low affinity benzoic acid transport system in this organism with K_m and V_{max} values of 0.72 μM and 3.3 nmol/min/mg cell dry wt and 5.3 μM and 4.6 nmol/min/mg cell dry wt, respectively.

Figure 5.1

(A) Effect of benzoic acid concentration on the initial rates of benzoic acid uptake by *Alcaligenes denitrificans* BRI 3010 under aerobic conditions.

(B) Double-reciprocal plot of initial rates of benzoic acid uptake by *Alcaligenes denitrificans* BRI 3010 under aerobic conditions.


A
v(nmol/min/mg cell dry wt)
1.4
1.2
1.0
0.8
0.6
0.4
0
10
20
30
Benzoic acid [μM]

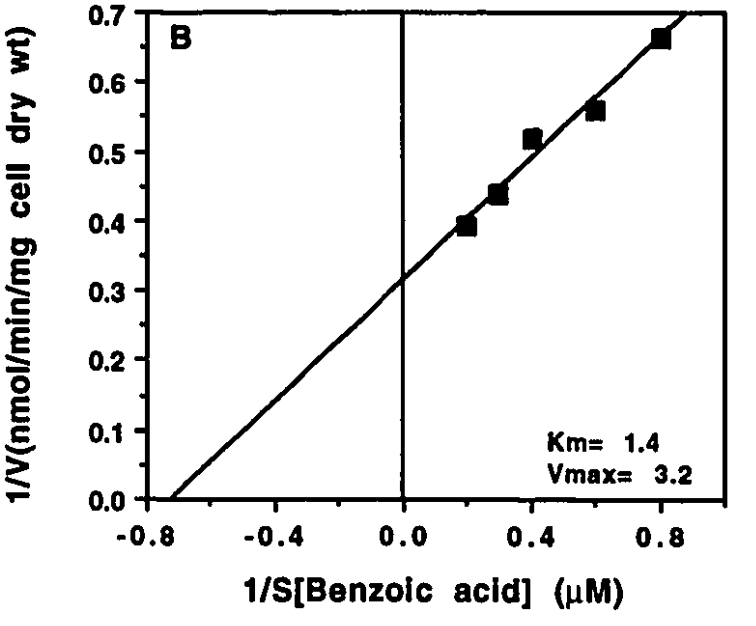
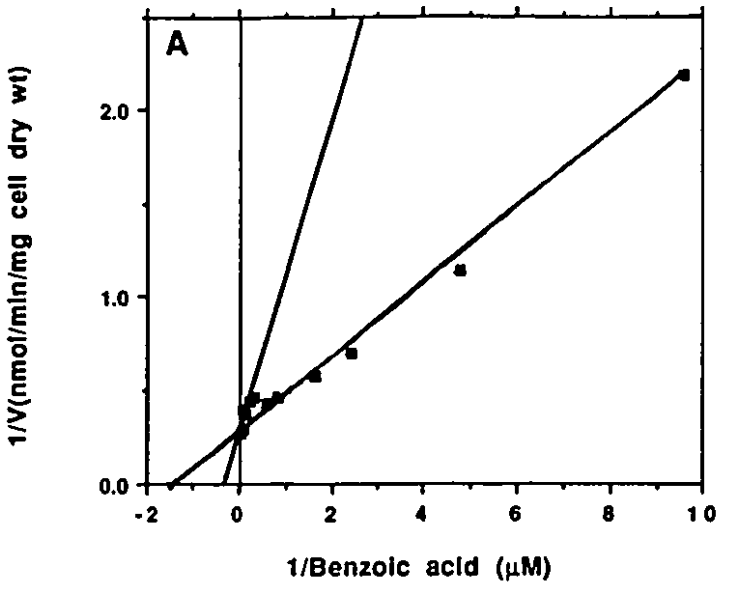
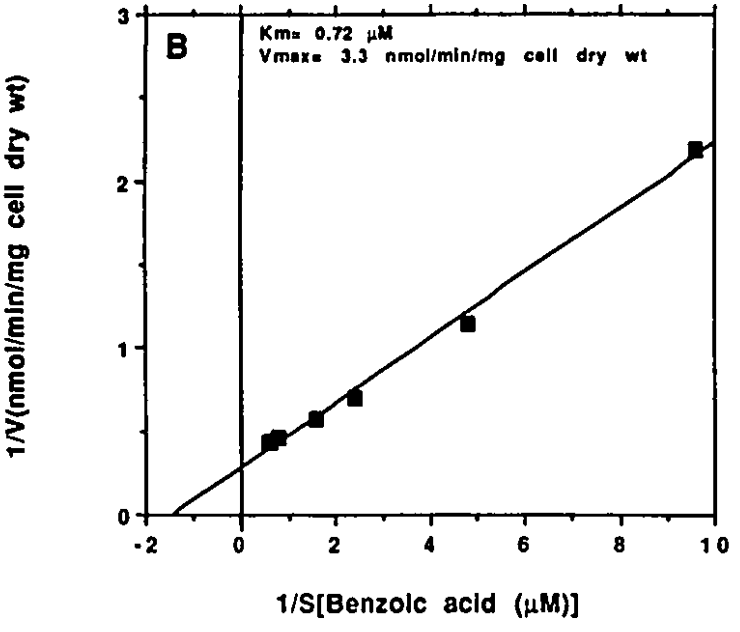
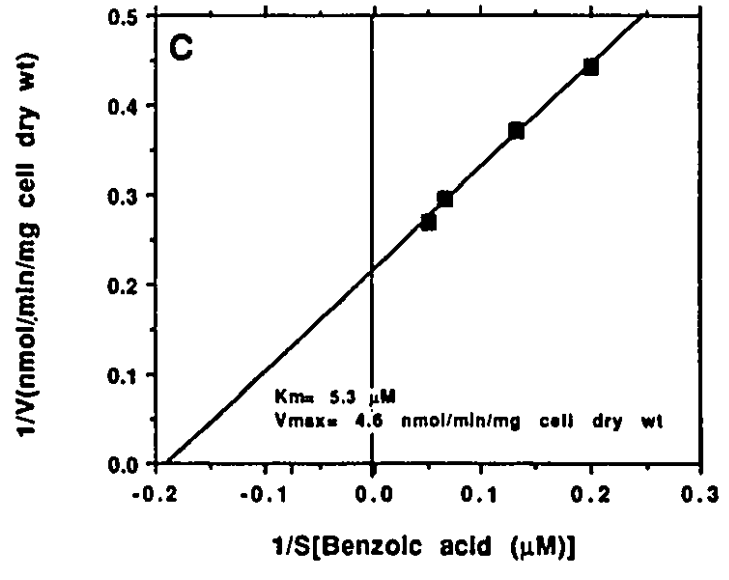

B
1/V(nmol/min/mg cell dry wt)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.8
-0.4
0.0
0.4
0.8
Km= 1.4
Vmax= 3.2
1/S[Benzoic acid] (μM)

Figure 5.2

Double-reciprocal plot of initial rates of benzoic acid uptake by *Alcaligenes denitrificans* BRI 6011 under aerobic conditions at concentrations ranging from 0.1 to 20.0 μM (A); 0.1 to 1.67 μM (B); and 5.0 to 20.0 μM (C).


A
1/V(nmol/min/mg cell dry wt)
2.0
1.0
0.0
-2
0
2
4
6
8
10
1/Benzoic acid (μM)


B
Km= 0.72 μM
Vmax= 3.3 nmol/min/mg cell dry wt
1/V(nmol/min/mg cell dry wt)
3
2
1
0
-2
0
2
4
6
8
10
1/S[Benzoic acid (μM)]


C
Km= 5.3 μM
Vmax= 4.6 nmol/min/mg cell dry wt
1/V(nmol/min/mg cell dry wt)
0.5
0.4
0.3
0.2
0.1
0.0
-0.2
-0.1
0.0
0.1
0.2
0.3
1/S[Benzoic acid (μM)]

When the initial rate of uptake of 2,4-DCBA by BRI 6011 was plotted against substrate concentration, a saturation curve of the Michaelis-Menten type was not obtained (Fig. 5.3A). In fact, the rate of uptake was found to be almost directly proportional to the concentration of substrate in the incubation medium. A double reciprocal plot of the data gave rise to a line passing through the origin thus confirming the absence of saturation kinetics (Fig. 5.3B).

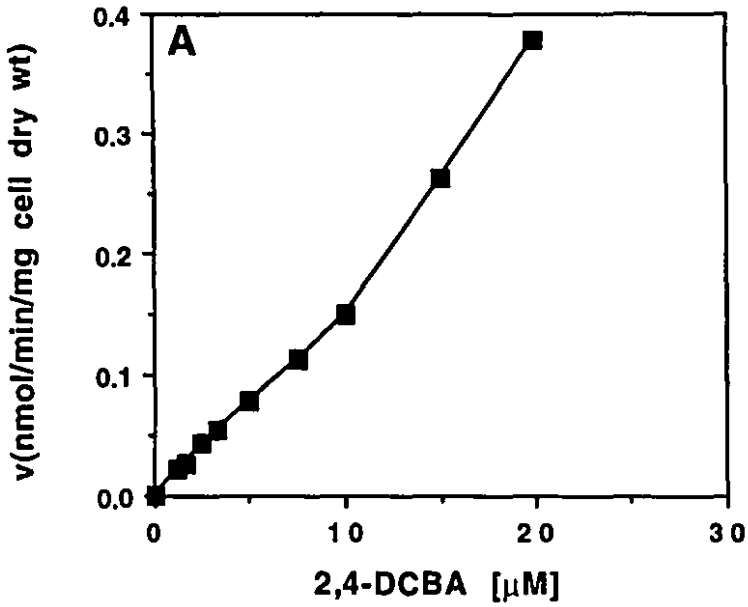
D: Evidence for active transport

In order to establish whether active transport of benzoic acid or of 2,4-DCBA had occurred, the ability of the cells to accumulate each compound in an unchanged state against a gradient was determined. For this purpose, the amount of radioactivity extracted by hot water as unchanged metabolite from a known weight of cells was determined. Assuming an intracellular fluid volume of 1.5 µl/mg cell dry wt (based on an average of 1.46 ± 0.125 µl/mg cell dry wt taken from published figures for eight other Gram negative rod forms with similar dimensions [Thayer and Wheelis 1982; Wong et al. 1991; Dr. MacLeod, personal communication]), the intracellular concentration of unchanged metabolite was calculated. The results reported in Table 5.3 show that both BRI 3010 and BRI 6011 concentrated benzoic acid inside the cells against a gradient to levels 8.5 and 10.4 fold, respectively, the external concentration. The percentage of radioactivity in the cells that was recovered as unchanged substrate after 4 min exposure to [^{14}C]-benzoic acid was low (3%), thus indicating a rapid conversion of benzoic acid into extractable and non-extractable metabolites in both BRI 3010 and BRI 6011. Indeed, 42% and 46% of the radioactivity remained associated with cell material after the hot water extraction of BRI 3010 and BRI 6011, respectively.

Figure 5.3

(A)Effect of 2,4-DCBA concentration on the initial rates of 2,4-DCBA uptake by *Alcaligenes denitrificans* BRI 6011 under aerobic conditions.

(B)Double-reciprocal plot of initial rates of 2,4-DCBA uptake by *Alcaligenes denitrificans* BRI 6011 under aerobic conditions at concentrations ranging from 1.67 to 20.0 μM


A
v(nmol/min/mg cell dry wt)
0.4
0.3
0.2
0.1
0.0
0
10
20
30
2,4-DCBA [μM]

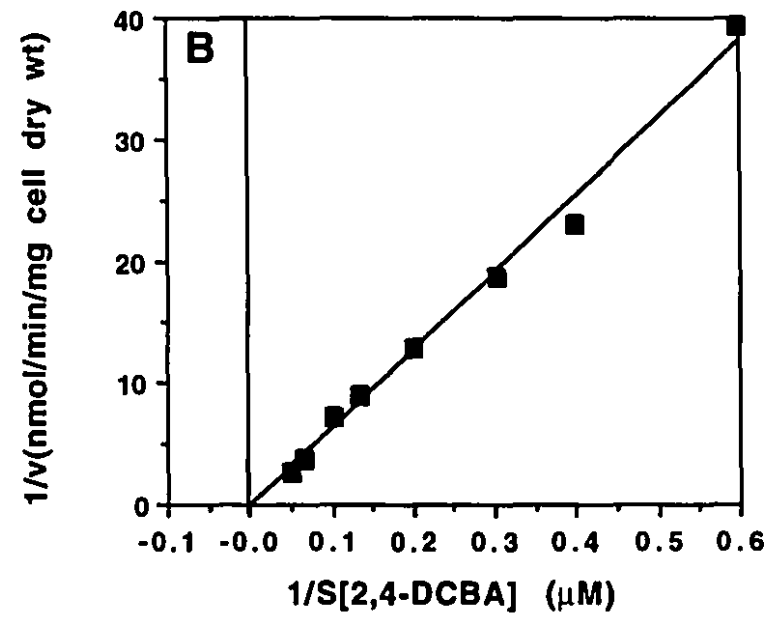

B
1/v(nmol/min/mg cell dry wt)
40
30
20
10
0
-0.1
-0.0
0.1
0.2
0.3
0.4
0.5
0.6
1/S[2,4-DCBA] (μM)

Table 5.3. Ability of *A. denitrificans* strains BRI 3010 and BRI 6011 to concentrate benzoic acid inside the cells against a gradient and failure of BRI 6011 to do the same with 2,4-DCBA

Metabolite taken up	Organism	I/E Ratio[a]	% Recovery[b]		
			as unchanged metabolite	as total extractable	as non-extractable
Benzoic acid	BRI 3010	8.5	3.0	58	42
	BRI 6011	10.4	3.1	54	46
2,4-DCBA	BRI 6011	0.6	0.4	30	70

[a] The I/E ratio is the ratio of intracellular concentration of unchanged metabolite (I) to initial extracellular concentration (E).

[b] %Recovery in each category is expressed as percent of total radioactivity present in the cells after incubation for 4 min in the presence of [^{14}C]-benzoic acid or 10 min in the presence of 2,4-DCBA. The substrates were each added as their K^+ salt.

Table 5.3 shows that BRI 6011 failed to concentrate 2,4-DCBA inside the cells against a gradient. After 10 min exposure of the cells to [^{14}C]-2,4-DCBA only 0.4% of the total radioactivity could be recovered as 2,4-DCBA while the rest had been converted to other extractable metabolites and non-extractable cell material.

E: Effect of metabolic inhibitors on uptake

The protonophore 3,5,3',4'-tetrachlorosalicylanilide (TCS) which destroys a proton motive force (Δp) and the respiratory inhibitor KCN inhibited uptake of benzoic acid by BRI 3010 and BRI 6011 almost completely and 2,4-DCBA uptake by BRI 6011 to a lesser extent (Table 5.4). Arsenate, an analogue of phosphate, which inhibits high energy phosphate bond formation had a limited inhibitory effect on benzoic acid uptake, but was more effective in reducing 2,4-DCBA uptake by BRI 6011. These observations suggest that uptake of benzoic acid is energized by a proton motive force (Δp). In an attempt to identify the component of the proton motive force responsible for driving uptake, the effect of the ionophores valinomycin and nigericin on the initial rate of uptake was assayed. Nigericin should mediate the exchange of external protons for internal K^+ and therefore abolish ΔpH across a membrane. This inhibitor effected marginal inhibition of benzoic acid and 2,4-DCBA uptake. Valinomycin which, in the presence of K^+ ions, should dissipate the membrane potential component of the Δp, was more inhibitory than nigericin for benzoic acid uptake but was marginally inhibitory to 2,4-DCBA uptake. The simultaneous addition of valinomycin and nigericin which should destroy the Δp, much like TCS (Racker 1976), had no more effect than valinomycin alone on the uptake of either compound.

Table 5.4. Effect of metabolic inhibitors on initial rates of uptake of benzoic acid and 2,4-DCBA

Inhibitor*	Organism†		
	BRI 3010	BRI 6011	BRI 6011
	% Inhibition of uptake of		
	Benzoic acid		2,4-DCBA
Na_2HAsO_4(10mM)	12.6 ± 7.2	17.7 ± 4.4	33.4 ± 4.1
TCS (10 μM)	87.2 ± 1.6	89.7 ± 1.3	77.2 ± 1.3
KCN (10 mM)	92.1 ± 1.5	94.4 ± 1.1	80.3 ± 2.4
Nigericin (5 μM)	17.2 ± 7.2	13.7 ± 7.4	12.1 ± 7.9
Valinomycin(10μM)	37.6 ± 4.5	39.5 ± 6.1	16.4 ± 8.2
Nigericin (5 μM) and Valinomycin(10μM)	39.2 ± 7.1	37.4 ± 6.6	17.1 ± 5.2

* Inhibitors were added to the suspension of cells in the incubation medium 15 min prior to the addition of the radioactive substrate.
† Cells were grown in minimal salts yeast extract medium supplemented with 5 mM benzoic acid. or 1 mM 2,4-DCBA for benzoic acid and 2,4-DCBA uptake studies, respectively. Results are presented as the average and average deviation of two determinations.
TCS= 3,5,3',4'-tetrachlorosalicylanilide

F: Effect of CBAs and other structurally related compounds on benzoic acid and 2,4-DCBA uptake

The effect of various chlorobenzoic acids and other compounds structurally related to benzoic acid on benzoic acid uptake was examined. With the exception of phenoxyacetic acid, all the compounds tested including those which are not metabolizable (Miguez et al. 1990), when added at a concentration 50-fold greater than that of the benzoic acid, significantly inhibited benzoic acid uptake by BRI 3010 and BRI 6011(Table 5.5). Similarly, all the compounds tested including those which are not metabolizable, inhibited 2,4-DCBA uptake significantly. Phenoxyacetic acid was not inhibitory. In contrast, benzoic acid uptake by *Pseudomonas* B13 was inhibited only by 3-CBA, the only CBA isomer that has been found to be metabolized by the organism.

In order to study the nature of the inhibition of benzoic acid uptake by BRI 3010 and BRI 6011 by structurally related compounds, the kinetics of inhibition were examined using 2,5-DCBA and 2-hydroxybenzoic acid (2-HBA) as inhibitors. A Cornish-Bowden plot (i.e., the benzoic acid concentration divided by the initial rate of benzoic acid uptake against the corresponding concentrations of the inhibitory analogue) was obtained for various 2,5-DCBA concentrations at benzoic acid concentrations of 5,10,15 and 20 μM for BRI 3010 (Figure 5.4), and for various 2-HBA concentrations at benzoic acid concentrations of 2.5, 5, 10 and 15 μM for BRI 6011 (Figure 5.5). The lines obtained by linear regression were convergent rather than parallel and thus not indicative of competitive inhibition (Cornish-Bowden 1974).

When the effect of 2-HBA on benzoic acid uptake by BRI 6011 was examined in more detail it was observed that at low concentrations, 2-HBA actually stimulated benzoic acid uptake. Only when the concentration of 2-HBA exceeded that of benzoic acid was inhibition obtained (Fig. 5.6). This

Table 5.5. Effect of various chlorobenzoic acids and other structurally related compounds on initial rates of benzoic acid and 2,4-DCBA uptake

Compound added	BRI 3010	BRI 6011	B13	BRI 6011
	% Inhibition of uptake of			
	Benzoic acid			2,4-DCBA
BA*†	95	96	94	83
2-CBA*	91	71	8	98
3-CBA†	87	65	80	99
4-CBA	78	72	11	N.D.
2,3-DCBA*	70	62	N.D.	N.D.
2,4-DCBA*	73	50	8	92
2,5-DCBA*	88	75	9	100
2,6-DCBA	55	41	11	100
3,4-DCBA	81	76	19	100
3,5-DCBA	84	82	N.D.	100
2,4-DFBA*	71	80	N.D.	84
2,5-DFBA*	90	77	10	N.D.
2-HBA*	95	96	16	93
2,4-DHBA*	54	40	8	78
2,5-DHBA*	56	N.D.	9	N.D.
3,4-DHBA*	53	N.D.	6	91
PA	0	0	7	33
2,4-D	47	55	0	95

Note: The unlabeled compound (1mM) was added to the incubation medium 1 min prior to the addition of ^{14}C-labeled benzoic acid or 2,4-DCBA (20 μM; 5 μCi /μmol). The percent inhibition of benzoic acid uptake was calculated from the initial rates of benzoic acid uptake in the presence and absence of each compound. Cells were grown to late log phase in minimal salts yeast extract medium supplemented with benzoic acid.

N.D.= Not determined; BA= Benzoic acid; PA= Phenoxyacetic acid; 2,4-D= 2,4-dichlorophenoxyacetic acid; 2-HBA= 2-hydroxybenzoic acid; DHBA= dihydroxybenzoic acid; DFBA= difluorobenzoic acid

*:Metabolized by BRI 3010 and BRI 6011, with the exception of 2,4-DCBA which is not metabolized by BRI 3010

†:Metabolized by *Pseudomonas* sp. B13

Figure 5.4

Kinetics of inhibition of benzoic acid uptake in *Alcaligenes denitrificans* BRI 3010 by 2,5-DCBA. Rates of uptake of benzoic acid (in nmoles/min/mg cell dry wt) were determined at the indicated concentrations of benzoic acid in the presence of increasing concentrations of 2,5-DCBA.

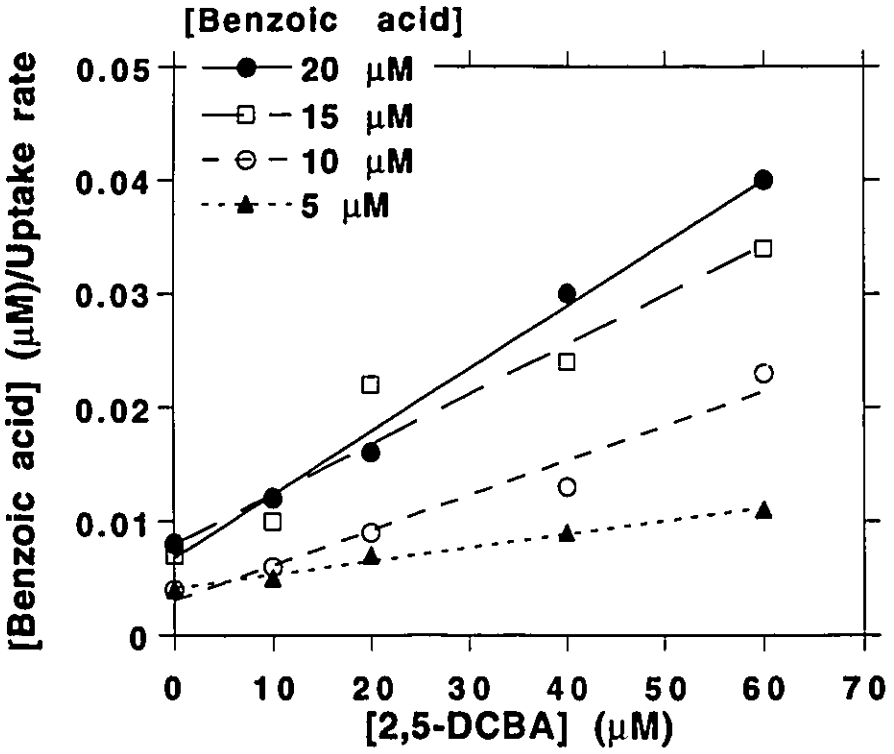

[Benzoic acid]
20 μM
15 μM
10 μM
5 μM
[Benzoic acid] (μM)/Uptake rate
0.05
0.04
0.03
0.02
0.01
0
0 10 20 30 40 50 60 70
[2,5-DCBA] (μM)

Figure 5.5

Kinetics of inhibition of benzoic acid uptake in *Alcaligenes denitrificans* BRI 6011 by 2-hydroxy-benzoic acid. Rates of uptake of benzoic acid (in nmoles/min/mg cell dry wt) were determined at the indicated concentrations of benzoic acid in the presence of increasing concentrations of 2-hydroxybenzoic.

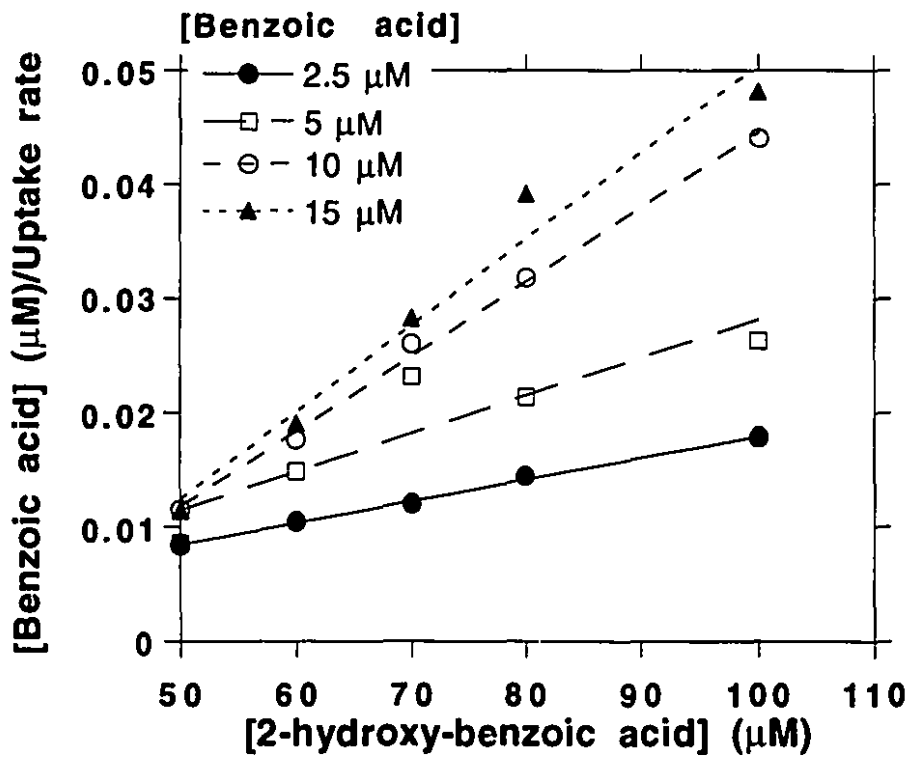
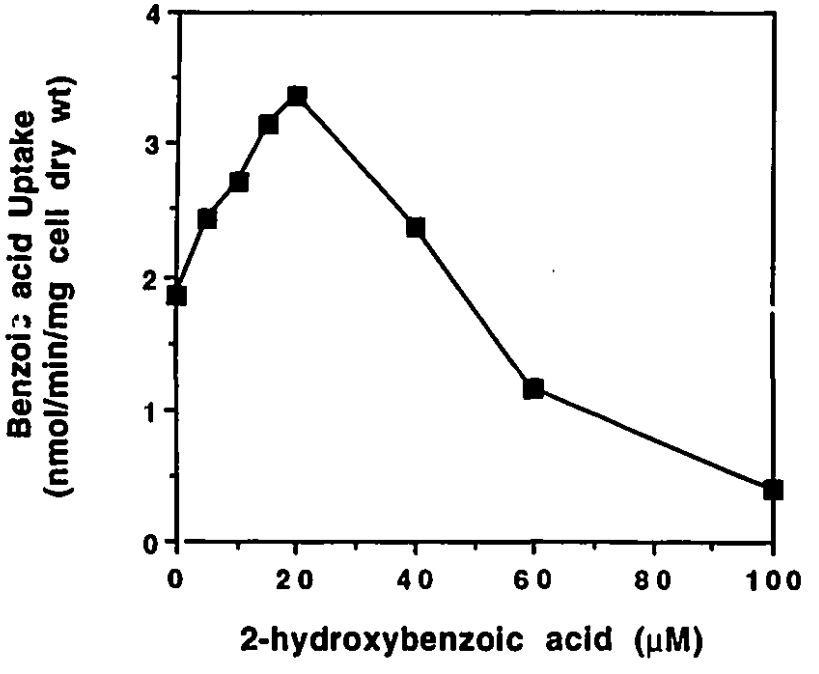

[Benzoic acid]
2.5 µM
5 µM
10 µM
15 µM
0.05
0.04
0.03
0.02
0.01
0
[Benzoic acid] (µM)/Uptake rate
50
60
70
80
90
100
110
[2-hydroxy-benzoic acid] (µM)

Figure 5.6

Effect of various concentrations of 2-hydroxy-benzoic acid on the initial rates of benzoic acid uptake by *Alcaligenes denitrificans* BRI 6011.


Benzoic acid Uptake
(nmol/min/mg cell dry wt)
0
1
2
3
4
0
20
40
60
80
100
2-hydroxybenzoic acid (μM)

stimulation of benzoic acid uptake in the presence of low concentrations of 2,4- and 2,5-DCBA was not observed.

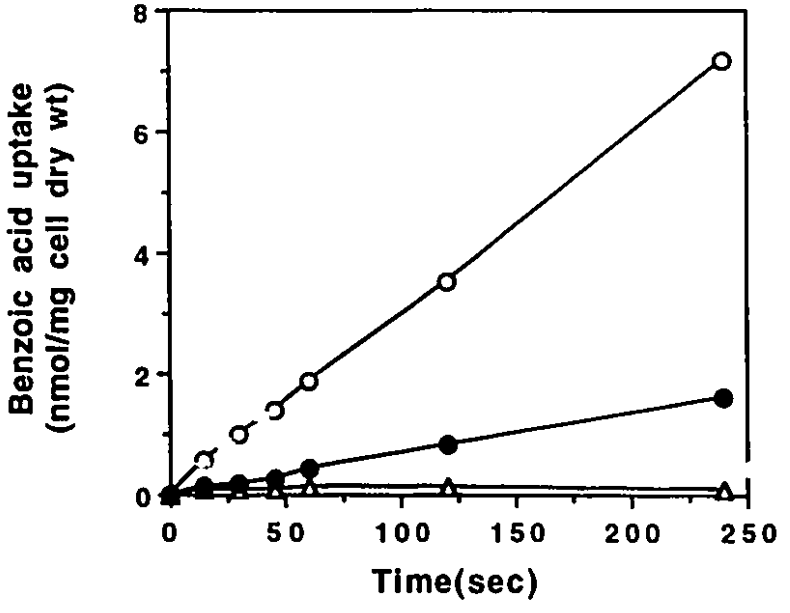
G: Effect of anaerobic conditions on uptake

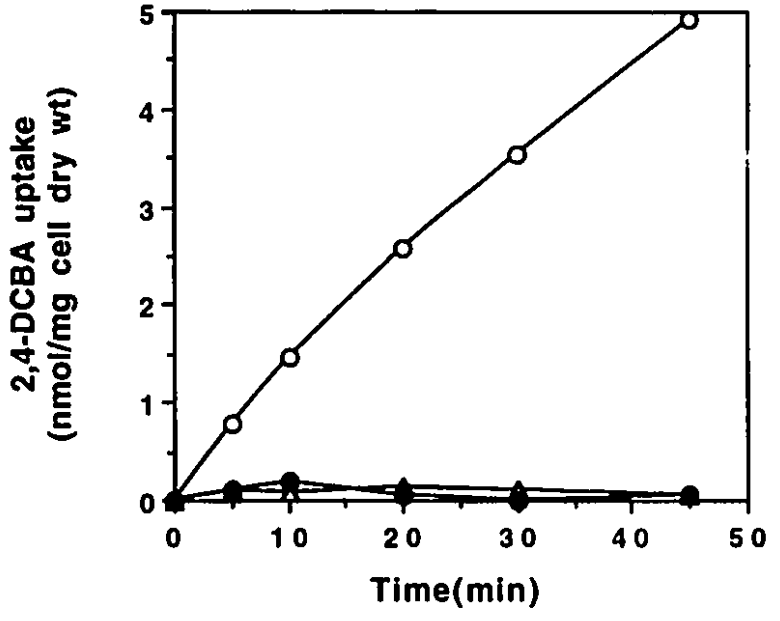
The ability of TCS and KCN to inhibit the uptake of benzoic acid and 2,4-DCBA so nearly completely indicates that energy is involved in the uptake process in the organisms tested. This is confirmed by the uptake results under anaerobic conditions (Fig. 5.7). BRI 6011 failed to take up benzoic acid in the absence of O_2 unless nitrate was present. The rate of uptake with nitrate, however, was much less than with O_2. Nitrate failed to substitute for O_2 for the uptake of 2,4-DCBA.

From the results in Fig. 5.7 it can be calculated that in the presence of O_2 the rate of uptake of benzoic acid into BRI 6011 is approximately 13 times faster that the rate of uptake of 2,4-DCBA.

Figure 5.7

Uptake of benzoic acid and 2,4-DCBA by *Alcaligenes denitrificans* BRI 6011 cells under aerobic (O) and anaerobic conditions in the absence (Δ) and presence of nitrate (●) (added as KNO_3, 60 mM final concentration). Cells were grown aerobically in the presence of benzoic acid (5 mM) for benzoic acid uptake experiments and in the presence of 1 mM 2,4-DCBA for 2,4-DCBA uptake. Uptake under anaerobic conditions was performed in an identical fashion to aerobic uptake experiments except that the incubation mixture was flushed continually with oxygen-free nitrogen gas. Cells were incubated for 30 minutes under these conditions prior to the addition of the ^{14}C-labeled substrate.


Benzoic acid uptake
(nmol/mg cell dry wt)
8
6
4
2
0
0
50
100
150
200
250
Time(sec)


2,4-DCBA uptake
(nmol/mg cell dry wt)
5
4
3
2
1
0
0
10
20
30
40
50
Time(min)

5.5 Discussion

The uptake of benzoic acid by BRI 3010, BRI 6011 and *Pseudomonas* sp. B13 was inducible. The rates of uptake of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 in response to different benzoic acid concentrations demonstrated saturation kinetics. These observations indicate a carrier mediated system for the uptake of benzoic acid into BRI 3010, BRI 6010, and perhaps into *Pseudomonas* sp. B13. Furthermore, although metabolism and uptake of benzoic acid by BRI 3010 and BRI 6011 occurred simultaneously, accumulation of the substrate to 8 and 10 times, respectively, the initial extracellular concentration was observed. This corresponds to an intracellular concentration of 170 μM and 208 μM for BRI 3010 and BRI 6011, respectively, and indicates that the transport process was an active one. These are relatively high values when considering that although benzoic acid was concentrated against a gradient, it was being rapidly converted to extractable and non-extractable metabolites and only 3% of the total radioactivity in the cell could be recovered as benzoic acid. *Pseudomonas putida* and two strains of *Rhizobium leguminosarum* accumulated benzoic acid and 3,4-DHBA against a 150- and 78-fold concentration gradient, respectively (Thayer and Wheelis 1982; Wong et al. 1991). However, in both of the above mentioned systems, the accumulation against a gradient occurred in mutated microorganisms defective in the metabolism of their respective substrate. Uptake of benzoic acid and 4-HBA by *Rhodopseudomonas palustris* grown anaerobically in light was also found to be energy dependent. However, intracellular accumulation of either substrate against a gradient was not observed. Both substrates are thioesterified by benzoyl- and 4-HBA-CoA ligase immediately upon entry into the cytoplasm. Consequently, simple diffusion of benzoic acid and 4-HBA, driven by a downhill

concentration gradient as a consequence of metabolism, was inferred (Harwood and Gibson 1986; Merkel et al. 1989).

The biphasic saturation kinetics observed when benzoic acid was taken up by BRI 6011, suggested the presence of two transport systems for benzoic acid with distinct substrate affinities. Although the monophasic saturation kinetics obtained with BRI 3010 suggests the presence of a single transport system for benzoic acid, this system had a high affinity for benzoic acid and was comparable to the high affinity benzoic acid transport system of BRI 6011. Dual transport systems are common and have been reported for the transport of branched-chain amino acids (Anraku et al. 1973; Fein and MacLeod 1975; Hoshino, 1979). However, dual transport systems for aromatic compounds are less well known. Evidence for two uptake systems in *Rhizobium leguminosarum* for two closely related compounds, 4-hydroxybenzoic (4-HBA) acid and 3,4-dihydroxybenzoic acid (3,4-DHBA), has been reported (Wong et al. 1991). Unfortunately, neither affinity constants of the postulated permeases nor their capability to bind and transport benzoic acid was reported. With regards to BRI 6011, it is possible that the low affinity transport system for benzoic acid may actually be a permease specific for another closely related compound.

Uptake of benzoic acid by BRI 3010 and BRI 6011 was energy dependent. Pre-incubation of the cells in the presence of a respirable substrate such as citrate, stimulated the initial rates of benzoic acid uptake. The ineffectiveness of arsenate as an inhibitor indicates that high energy phosphate bond formation is not a driving force for the transport of benzoic acid by BRI 3010 and BRI 6011. Lack of benzoic acid uptake anaerobically or aerobically in the presence of either the respiratory inhibitor, KCN, or the protonophore, TCS, is indicative of a proton motive force being involved in the uptake system. The

component of the Δp responsible for driving benzoic acid uptake by BRI 3010 and BRI 6011 is not known. The addition of valinomycin and nigericin resulted in partial and negligible inhibition of benzoic acid uptake, respectively. The simultaneous addition of both inhibitors, which should uncouple the system, contradicted the inhibitory effects of TCS. The ineffectiveness of the inhibitory action of valinomycin and nigericin may be attributable to a decreased penetrability of the outer membrane to these inhibitors. MacLeod et al. (1988) have shown that some Gram-negative bacteria exhibit varying sensitivities to protonophores which may be attributable to differences in the permeability of the outer membrane. Before speculating further on the susceptibility of BRI 3010 and BRI 6011 to these inhibitors, the effect of nigericin and valinomycin on the uptake of other transportable carbon sources should be tested.

In contrast to the uptake of benzoic acid by BRI 6011, uptake of 2,4-DCBA by the same organism was not inducible nor was there evidence of an active uptake of 2,4-DCBA. The slow rate of entry of 2,4-DCBA and the lack of saturation kinetics point to entry via diffusion through a lipid membrane down a concentration gradient. Also, in a similar fashion to the non-carrier mediated uptake of benzoic acid and 4-HBA by *Rhodopseudomonas palustris* (Harwood and Gibson 1986; Merkel et al. 1989) and 2,4-D by *Pseudomonas fluorescens*. (Wedemeyer 1966), uptake of 2,4-DCBA is inhibited by the respiratory inhibitor KCN and partially by arsenate. This, in addition to the inhibition of 2,4-DCBA uptake by the uncoupler TCS, is suggestive of an energy dependent uptake process. From what is known concerning the uptake of lipophilic weak acids, we presume that 2,4-DCBA diffuses through the lipid membrane in an undissociated form driven inward by a ΔpH inside alkaline. If the diffusable compound is metabolized upon entry into the cytoplasm, energy requiring metabolic-drag then becomes an essential driving force for uptake. Evidence,

implicating both ΔpH and metabolic drag as driving forces of 2,4-DCBA uptake is presented. Only 0.4% of total radioactivity in the cell was recovered as 2,4-DCBA, indicating extensive metabolism of the substrate thereby maintaining a downhill gradient into the cell. The stimulation of 2,4-DCBA uptake by an alternate respirable carbon source and the strong inhibition of uptake imparted by structurally related lipophilic weak acids, is indirect evidence implicating ΔpH inside alkaline as the driving force for uptake. In fact, the inhibition of 2,4-DCBA uptake in the presence of structurally related compounds was higher than the inhibition of benzoic acid uptake by the same compounds. Phenoxyacetic acid which did not inhibit benzoic acid uptake, partially inhibited 2,4-DCBA. These lipophilic weak acids, at a 50-fold concentration excess, may provide sufficient influx of protons to abolish the ΔpH in the cells. The increased rates of 2,4-DCBA uptake by cells grown in minimal salts yeast extract medium over that of cells grown in the same medium supplemented with 2,4-DCBA is rather perplexing. It is possible that the presumed low and transient intracellular concentrations of 2,4-DCBA in 2,4-DCBA grown cells, may actually regulate the subsequent uptake of the substrate. The ability of a transport system to differentiate between an aromatic compound and its chlorinated analogue is remarkable but not unique. The carrier mediated uptake of 4-CBA by the coryneform bacterium NTB-1 is inhibited mainly by *para*-substituted analogues of benzoic acid. Benzoic acid marginally inhibits 4-CBA uptake by NTB-1 and therefore is not expected to share a common carrier protein.

The profile of benzoic acid uptake inhibition by a wide range of structurally related metabolizable and non-metabolizable compounds, suggests that the membranes of BRI 3010 and BRI 6011 are very penetrable by the lipophilic weak acids studied. Although 2,4-DCBA inhibits the uptake of benzoic acid by BRI 6011, the inhibition can not be at the level of the benzoic

acid carrier protein, since as previously discussed there is no correlation between the induction of the saturable benzoic acid transport system and the uptake of 2,4-DCBA by the same organism. In an attempt to gain some insight into the nature of the inhibition imparted by these analogues, kinetic analysis of the inhibition of benzoic acid uptake by 2-HBA and 2,5-DCBA was studied, and revealed the inhibition not to be competitive. This reinforces the belief that at sufficiently high concentrations, these lipophilic weak acids act as protonophores. At low concentrations, 2-HBA which is a metabolizable substrate, diffuses across the membrane and is subsequently metabolized, thereby stimulating respiration and benzoic acid uptake. However, at higher concentrations, 2-HBA diffuses into the cells at a sufficient rate and concentration to dissipate the Δp and subsequently uncouple benzoic acid uptake. In the absence of direct measurements of the membrane potential and ΔpH in cells in response to increasing concentrations of inhibitory analogues, these observations remain speculative.

In contrast to BRI 3010 and BRI 6011, only 3-CBA effectively inhibited benzoic acid uptake by *Pseudomonas* sp. B13 and the membranes of this organism appeared to be considerably less permeable to lipophilic weak acids. This rather specific inhibition by a metabolizable substrate appears to suggest a common carrier for both benzoic acid and 3-CBA in *Pseudomonas* sp. B13. However, 3-CBA is an efficient inhibitor of benzoic acid dioxygenation and therefore the elucidation of a permease in *Pseudomonas* sp. B13 for benzoic acid must first be established before a definitive statement on the specificity of the uptake system can be made. Reineke and Knackmuss (1978a) measured relative rates of dioxygenation of benzoic acid and substituted benzoic acids by whole cells of *Pseudomonas* sp. B13 and observed that at equimolar concentrations, 3-CBA inhibited benzoic acid turnover by 30%.

The cell membranes of BRI 3010 and BRI 6011 are very permeable to lipophilic weak acids, including 3-CBA. We can not therefore implicate uptake as a contributing factor in the inability of BRI 3010 and BRI 6011 to metabolize 3-CBA, or the inability of BRI 3010 to metabolize 2,4-DCBA. These differences in substrate specificities appear to be attributable to the different specificities of a benzoic acid dioxygenase in the organisms.

Section 6

Oxidation and utilization of chlorobenzoic acids by *Alcaligenes denitrificans* BRI 3010 and BRI 6011

6.0 Preface

This section sheds light on the mechanism of initial substrate dioxygenation by studying oxygen uptake responses by whole cells to various substituted benzoic acids, and the role this might have in differentiating BRI 3010, BRI 6011, and *Pseudomonas* sp. B13, with respect to their CBA substrate versatility. In a contaminated environment, microbial populations typically encounter heterogeneous mixtures of organic compounds. Results presented in section 3 of this thesis, revealed that when 2,4- and 2,5-DCBA were present as mixed substrates in the growth medium, sequential utilization was observed where 2,5-DCBA was markedly the preferred substrate. This section describes the sequential utilization pattern of metabolizable mixtures of CBAs in order to address the potential efficacy of CBA biodegradation by BRI 6011 in the environment.

6.1 ABSTRACT

Oxygen consumption studies with whole cells of *A. denitrificans* strains BRI 3010 and BRI 6011 in the presence of benzoic and substituted benzoic acids were performed. Benzoic acid-grown cells exhibited increased rates of benzoic acid oxidation relative to CBA grown cells. In contrast, growth on CBAs resulted in increased rates of CBA oxidation. Growth of both organisms on 2-CBA resulted in the stoichiometric release of chloride ions and the induction of catechol 1,2-dioxygenase instead of chlorocatechol 1,2-dioxygenase. These findings suggest that in addition to a benzoic acid dioxygenase, a halobenzoic acid dioxygenase is operative in the degradation of 2-CBA by BRI 3010 and BRI 6011. This enzyme, presumably a 2-chlorobenzoic acid 1,2-dioxygenase, appears to also catalyze the oxygenation of the *ortho*-substituted DCBAs utilized by both BRI 3010 and BRI 6011.

BRI 6011 grown on different CBAs, individually present in the growth medium, utilized the substrates in the following decreasing order of preference: 2-CBA, 2,5-, 2,4-, and 2,3-DCBA. In mixed substrate growth conditions, sequential utilization of the CBAs was observed.

These results indicate that BRI 3010 and BRI 6011 are well adapted for the degradation of CBAs and may potentially play an important role in the bioremediation of selected aromatic compounds.

6.2 Introduction

The microbial degradation of chlorinated biphenyls (CBPs) in the environment is very slow and is generally dependent on the existence of a microbial population with biphenyl (BP) degradative capabilities which can co-metabolize the CBP substrate to the corresponding chlorinated benzoic acid(s) (CBAs). Chlorobenzoic acids will accumulate in the environment in the absence of a microbial population with CBA degradative capabilities. Complete mineralization of PCBs by natural axenic isolates has not been reported. This has only been achieved by mixed cultures (co-cultures) consisting of a CBA degrader in conjunction with a BP-CBP degrader. Such associations have only been reported to degrade congeners that produce 4-CBA as the end product of the BP-CBP degradation pathway by the CBP-degrading member (Adriaens et al. 1989; Pettigrew et al. 1990; Sylvestre et al. 1985). This metabolic constraint may be attributed to incompatibility of the BP and CBA degradative pathways (Sondossi et al. 1992). It was observed that 3-chlorocatechol (3-CC) efficiently inhibited the activity of the 2,3-dihydroxybiphenyl 1,2-dioxygenase, the *meta*-cleavage enzyme of the BP pathway in *Pseudomonas testosteroni*. Catalytic attack of 3-CC by the *meta*-cleavage enzyme forms 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid which is a strong inhibitor of the enzyme (Bartels et al. 1984; Klecka and Gibson 1981; Reineke and Knackmuss 1980). The *meta*-cleavage enzyme was also inhibited by 4-CC, but to a much lesser extent. The formation and subsequent metabolism of other chlorinated benzoic acids via 3-CC or 4-CC will thereby inhibit BP ring cleavage and consequently limit the extent of BP and CBP degradation. Similarly, incompatibility of the BP and CBA degradative pathways within a 3-CBP-utilizing recombinant strain, *Pseudomonas* sp. CB15, has been reported. In addition to the inhibition of 2,3-dihydroxybiphenyl 1,2-dioxygenase by 3-CC in this organism, 3-chloro-2',3'-

dihydroxybiphenyl inhibited catechol 1,2-dioxygenase activity competitvely (Adams et al. 1992). The co-cultures capable of degrading 4-CBP, form 4-CBA as the CBA intermediate which is subsequently metabolized via 4-hydroxybenzoic acid (4-HBA) and protocatechuic acid (3,4-DHBA) thus avoiding the inhibitory effect of chlorinated catechols.

In addition to the CBAs formed as a consequence of the aerobic degradation of PCBs, CBAs are also introduced into the environment via the application of herbicides such as 2,5-dichloro-3-aminobenzoic acid, 2,3,6-trichlorobenzoic acid and 2,6-dichlorobenzonitril (Horvath, 1972). Efficient removal of this heterogeneous mixture of CBAs requires the presence of chlorobenzoic acid degraders with broad substrate specificity, of which few have been isolated. Two natural isolates with expanded chlorobenzoic acid substrate versatility capable of degrading di- , and trichlorobenzoic acids have recently been reported (Hickey and Focht 1990; Adriaens and Focht 1991). Also, aerobic degradation of the notoriously recalcitrant *ortho*-substituted benzoic acids by natural isolates has been described. *Pseudomonas cepacia* 2CBS, converts 2-CBA directly to catechol which is subsequently subjected to *meta*-cleavage (Fetzner et al. 1989a, 1989b, 1992). Similarly, *P. putida* P111 converts *ortho*-substituted dichlorobenzoic acids directly to the corresponding catechols. However, in this organism the chlorinated catechols are subjected to *ortho*-cleavage by the chlorocatechol 1,2-dioxygenase (Hernandez et al. 1991). In both of the above mentioned cases, the conversion of substituted benzoic acid directly to the corresponding catechol obviates the need for a functional dihydrodiol dehydrogenase and exemplifies the diversity in aerobic chlorobenzoic acid degradation patterns.

Alcaligenes denitrificans BRI 3010 and BRI 6011 are chlorobenzoic acid degrading microorganisms, capable of growth on 2-CBA, 2,3- , and 2,5-DCBA.

BRI 6011 also degrades 2,4-DCBA. This study investigated the effect that growth on mixed chlorobenzoic acid substrates had on mixed substrate competition and inhibition in BRI 6011, which is a growing concern when contemplating *in situ* bioremediation of chloroaromatic contaminated environments (Hernandez et al. 1991; Haigler et al. 1992; Fox 1992). Also, in an attempt to gain some insight into the mechanism(s) of the initial substrate dioxygenation, the initial rates of oxidation of benzoic and substituted benzoic acids by cells grown on various substituted benzoic acids was investigated.

6.3 Materials and Methods

Organisms

The isolation and characterization of *Alcaligenes denitrificans* strains BRI 3010 and BRI 6011 have been previously described (Miguez et al. 1990).

Single and mixed substrate growth conditions.

A. denitrificans BRI 6011 was grown in minimal salts medium (MSM), supplemented with yeast extract (100 mg/l). The chlorobenzoic acid isomers, were used at concentrations of 0.5-0.6 mM. The culture was grown in Erlenmeyer flasks incubated at 30°C, and 250 rpm. At the indicated sampling times, 2.5 ml of culture was removed for optical density (660 nm) measurements and following centrifugation (Eppendorf), for analysis of the supernatant (1.2 ml) for substrate by HPLC.

Preparation of resting cells and cell lysates

Cells grown in 500 ml MSM containing 100 mg/l Y.E. and 0.5 mM benzoic acid (BA) or the respective chlorobenzoic acids, were recovered by centrifugation (10,000 x g, 20 min, 4°C) in the late exponential growth phase,

washed twice with sterile phosphate buffer (10 mM, pH 7.0), and subsequently resuspended in sterile phosphate buffer to give resting cell suspensions. Cell lysate was prepared as described previously (Miguez et al. 1990).

Oxidation of aromatic compounds by resting cells and by cell lysates

Oxygen uptake was measured at 30°C using a Clark-type oxygen electrode (model 5300, Yellow Springs Instrument Co., Yellow Springs, Ohio). A 3-ml reaction mixture contained resting cells in potassium phosphate buffer (10 mM, pH 7.0) at an approximate O.D. $_{660\ nm}$ of 0.2 (corresponding to 0.25 to 0.30 mg protein). The reaction was started by injecting 30 µl of 10 mM substrate dissolved in methanol. Oxygen consumption rates were corrected for endogenous respiration, which was unaffected by methanol alone. Oxidation of catechol and chlorocatechols by cell lysates has been previously described (Miguez et al. 1993).

Benzoic acid and chlorobenzoic acid dioxygenase activity

Dioxygenase activity was assayed at 30°C spectrophotometrically at 340 nm (NADH consumption) and polarographically (oxygen consumption) as described above. The reaction mixture contained 100 mM ammonium acetate buffer pH 6.6, cell lysate (approximate final protein concentration, 3.0 mg/ml), 70 µM $(NH_4)_2Fe(SO_4)_2$, 0.2 mM NADH, and 0.2 mM benzoic acid or chlorobenzoic acids dissolved in methanol.

Analytical

Benzoic acid and chlorinated benzoic acids were determined by HPLC as previously described (Miguez et al. 1990). Inorganic chloride was determined colorimetrically (Florence and Farrar 1971), using a sample volume

of 1.0 ml, and sodium chloride as standard. The Lowry method (Markwell et al.1978), was used to estimate protein concentrations with bovine serum albumin as standard. The source of aromatic and chloro-aromatic compounds has been previously described (Miguez et al. 1990).

6.4 Results

A: Oxidation of aromatic compounds by cell suspensions of BRI 3010 and BRI 6011

Rates of oxygen uptake by whole cells were determined in order to ascertain the relative rates of metabolism of benzoic acids and catechols, and also to investigate the effect of induction on these rates. Diminished rates of benzoic acid oxidation were observed in BRI 6011 and BRI 3010 grown on chlorinated benzoic acids (Table 6.1) and (Table 6.2), respectively. In contrast, rates of chlorinated benzoic acids oxidation increased in cells grown on these chlorinated substrates. The oxidation rates of the chlorobenzoic acids remained relatively constant in chlorobenzoic acid grown cells, except for the moderate increase in the oxidation rate of 2-CBA and 2,5-DCBA in 2,5-DCBA grown cells. Oxidation of 2,4-DCBA by BRI 3010 was not observed, regardless of previous growth conditions. The oxidation rate profile of catechols and substituted catechols was similar in cells grown on benzoic acid and or 2-CBA (Table 6.1). Growth on these substrates induced pyrocatechase activity mainly against catechol, implicating the action of the catechol 1,2-dioxygenase enzyme. The increased oxidation rates of all catechols were observed in cells grown on 2,4- and 2,5-DCBA, implicating the induction of the chlorocatechol 1,2-dioxygenase (Table 6.1). The rates of 3,5- and 3,6-DCC oxidation by 2,4- and 2,5-DCBA grown cells were significantly lower than that of 4-CC. However, cell extracts of BRI 6011 grown on 2,4- and 2,5-DCBA (Table 6.3) oxidized

Table 6.1. Oxygen consumption by washed cells of BRI 6011

Assay substrate	Oxygen consumption [nmol/min/mg protein] after growth with			
	BA	2-CBA	2,4-DCBA	2,5-DCBA
BA	72 ± 4	31 ± 2	38 ± 1	36 ± 1
2-HBA	175 ± 11	32 ± 3	25 ± 0	47 ± 1
2,5-DHBA	62 ± 5	22 ± 3	9 ± 1	34 ± 3
2-CBA	40 ± 3	91 ± 5	84 ± 2	108 ± 2
2,3-DCBA	9 ± 2	30 ± 2	38 ± 2	42 ± 2
2,4-DCBA	12 ± 2	58 ± 3	61 ± 3	71 ± 2
2,5-DCBA	18 ± 3	61 ± 3	64 ± 2	93 ± 3
Catechol	60 ± 4	76 ± 4	149 ± 5	100 ± 2
4-CC	12 ± 1	18 ± 1	139 ± 4	97 ± 2
3,5-DCC	0	0	13 ± 2	31 ± 1
3,6-DCC	0	0	28 ± 1	52 ± 4
3-Met-C	25 ± 6	32 ± 2	156 ± 5	145 ± 5
4-Met-C	27 ± 4	38 ± 2	154 ± 4	113 ± 3

No activity was observed with the following substrates: 3-CBA, 4-CBA, 2,6-DCBA, 3,4-DCBA, 3,5-DCBA, phenol, and chlorinated phenols.
Oxygen consumption rates are the mean and average deviation of duplicate or triplicate assays.

Table 6.2. Oxygen consumption by washed cells of BRI 3010

Assay substrate	Oxygen consumption [nmol/min/mg protein] after growth with		
	BA	2-CBA	2,5-DCBA
BA	83 ± 2	36 ± 3	41 ± 2
2-CBA	36 ± 2	89 ± 2	95 ± 3
2,5-DCBA	22 ± 1	74 ± 2	88 ± 2

No activity was observed with the following substrates:3-CBA, 4-CBA, 2,4-DCBA, 2,6-DCBA, 3,4-DCBA, 3,5-DCBA, phenol, and chlorinated phenols. Oxygen consumption rates are the mean and average deviation of duplicate or triplicate assays.

Table 6.3. Oxygen consumption by cell extracts of BRI 6011

Assay substrate	Oxygen consumption [nmol/min/mg protein] after growth with		
	BA	2,4-DCBA	2,5-DCBA
Catechol	40 ± 1	61 ± 2	52 ± 1
4-CC	7 ± 2	59 ± 1	54 ± 2
3,5-DCC	9 ± 2	55 ± 2	56 ± 1
3,6-DCC	7 ± 1	58 ± 3	41 ± 2

Oxygen consumption rates are the mean and average deviation of duplicate or triplicate assays.

all chlorinated catechols at the same relative rate. This difference in the relative rates of oxidation between whole cells and cell lysates may be attributable to permeability differences of these substrates.

Previous studies have shown that BRI 6011 utilized salicylic acid (2-HBA) as a carbon source (section 3 of this thesis), and that it served as an energy source in the uptake of benzoic acid (section 5 of this thesis). Table 6.1 reveals that benzoic acid grown cells oxidized salicylic acid at a greater rate than benzoic acid. Gentisic acid (2,5-DHBA), also utilized as a carbon source by BRI 6011, was oxidized at a comparable rate to benzoic acid in benzoic acid grown cells.

Accurate measurements of benzoic acid dioxygenase activity by polarographic and photometric assays were not possible due to the high endogenous NADH consumption and oxygen uptake by the crude cell lysate. This phenomenon has also been observed in crude lysates of *P. cepacia* 2CBS (Fetzner *et al.* 1992).

B: Utilization of chlorobenzoic acid isomers by A. denitrificans BRI 6011.

The time required for complete utilization of the chorobenzoic acid isomers when present individually in the growth medium, differed significantly (Figure 6.1). The utilization, in decreasing order of preference, was: 2-CBA, 2,5-, 2,4-, and 2,3-DCBA. When the substrates were mixed in the growth medium, sequential substrate utilization was observed. When 2-CBA and 2,4-DCBA were present in the growth medium simultaneously, total consumption of 2,4-DCBA was delayed by approximately 5 hrs. Utilization of 2,4-DCBA by the culture began only when most of the 2-CBA in the growth medium had been depleted, whereas the rate of 2-CBA utilization remained unchanged (Figure 6.2). Similarly, when 2-CBA and 2,5-DCBA were present

Figure 6.1

Utilization of chlorobenzoic acids present individually in the growth medium at the indicated concentrations by BRI 6011. For assay conditions see Materials and Methods.

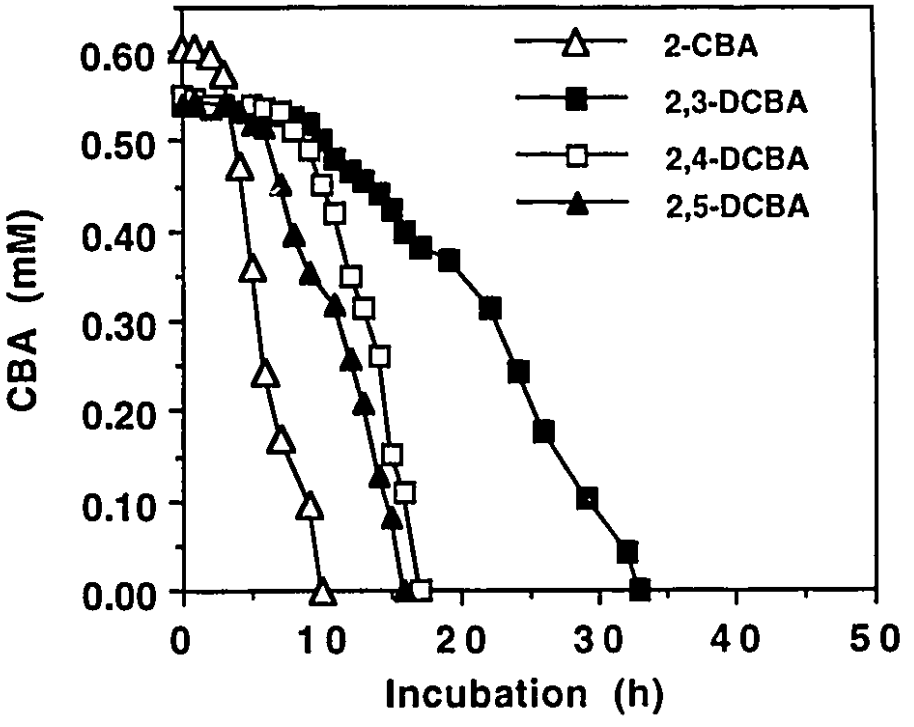
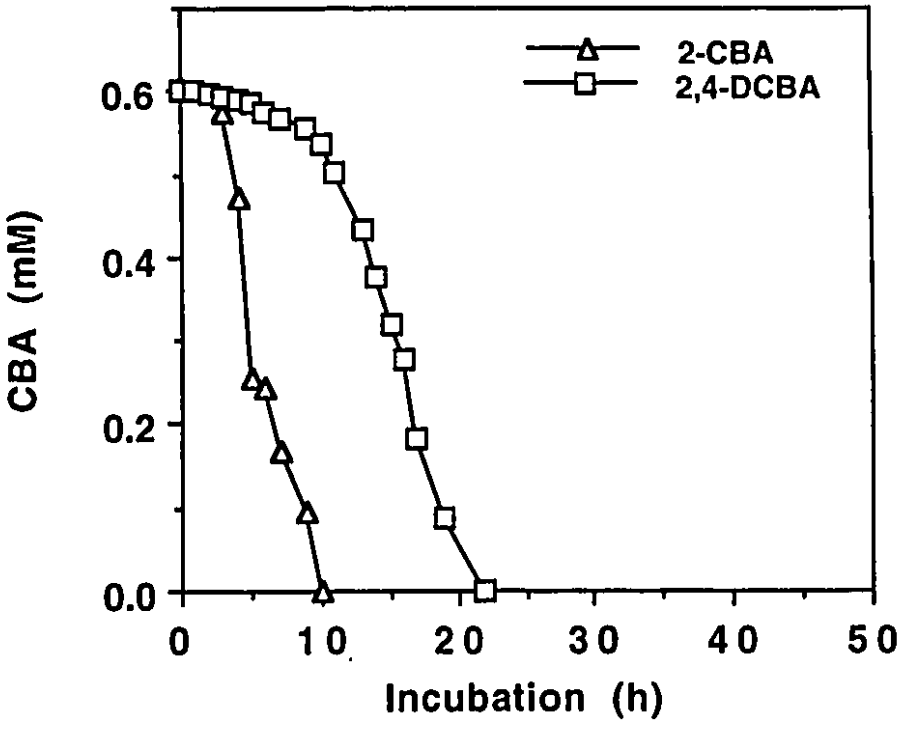

2-CBA
2,3-DCBA
2,4-DCBA
2,5-DCBA
CBA (mM)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
Incubation (h)

Figure 6.2

Utilization of 2-CBA and 2,4-DCBA present in the growth medium simultaneously at the indicated concentrations by BRI 6011. For assay conditions see Materials and Methods.


2-CBA
2,4-DCBA
CBA (mM)
0.0
0.2
0.4
0.6
0
10
20
30
40
50
Incubation (h)

simultaneously in the growth medium, 2-CBA utilization remained unchanged but its presence delayed 2,5-DCBA utilization by the culture. When 2,4- and 2,5-DCBA were together in the growth medium, consumption of 2,4-DCBA was delayed by approximately 10 hrs, whereas the 2,5-DCBA utilization rate remained unaffected (Figure 6.3). When 2,3- and 2,4-DCBA were present together in the growth medium, simultaneous utilization of these two substrates was observed (Figure 6.4). Complete 2,3- and 2,4-DCBA utilization was delayed by 6 and 23 hrs, respectively, when present together. Inclusion of 2,5-DCBA to this substrate combination (Figure 6.5), resulted in a greater delay of 2,3- and 2,4-DCBA utilization. Occasionally and unexpectedly, the presence of 2,3- and 2,4-DCBA in the growth medium simultaneously, resulted in incomplete utilization of both substrates. In all cases mentioned above, substrate utilization coincided with the stoichiometric release of chloride. The culture used as the inoculum for all of the substrate utilization experiments was grown on a mixed substrate medium containing 2-CBA, 2,3- , 2,4- , and 2,5-DCBA, and consequently presumed to be metabolically fully induced.

Figure 6.3

Utilization of 2,4-DCBA and 2,5-DCBA present in the growth medium simultaneously at the indicated concentrations by BRI 6011. For assay conditions see Materials and Methods.

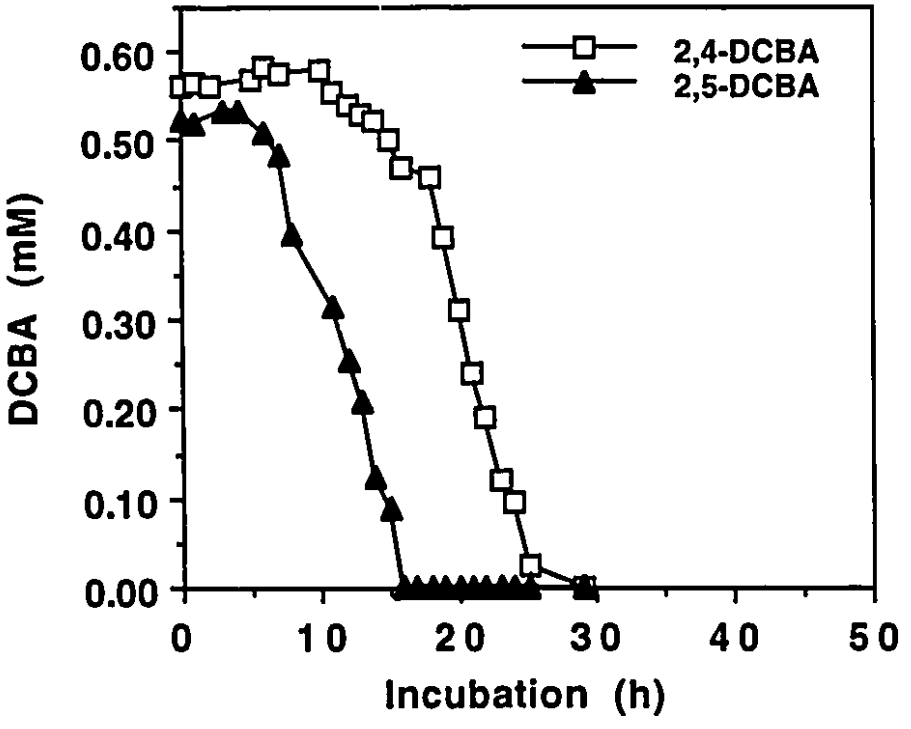

2,4-DCBA
2,5-DCBA
DCBA (mM)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
Incubation (h)

Figure 6.4

Utilization of 2,3-DCBA and 2,4-DCBA present in the growth medium simultaneously at the indicated concentrations by BRI 6011. For assay conditions see Materials and Methods.

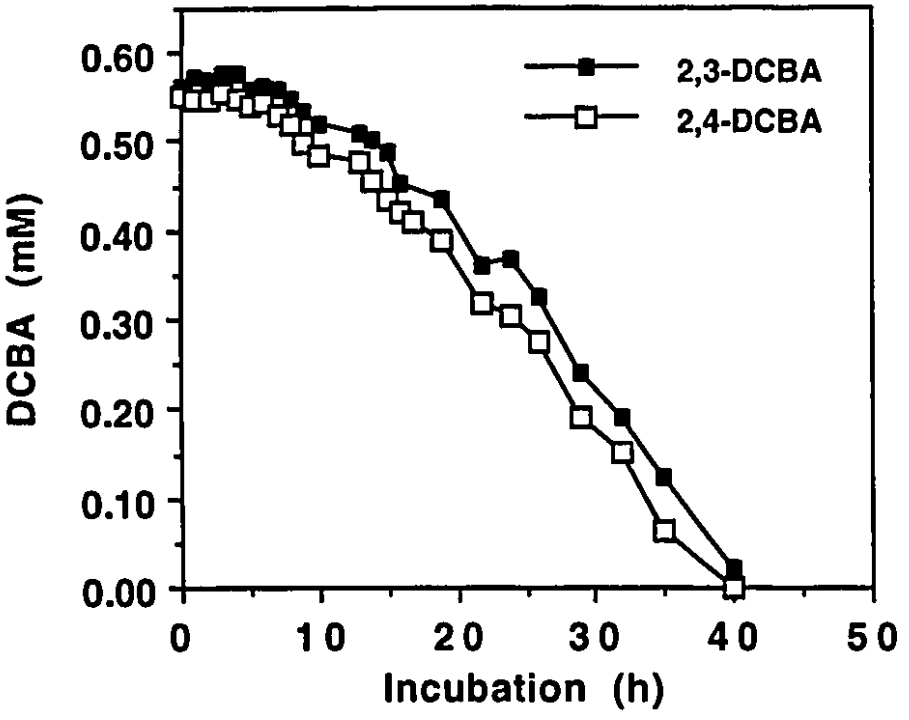
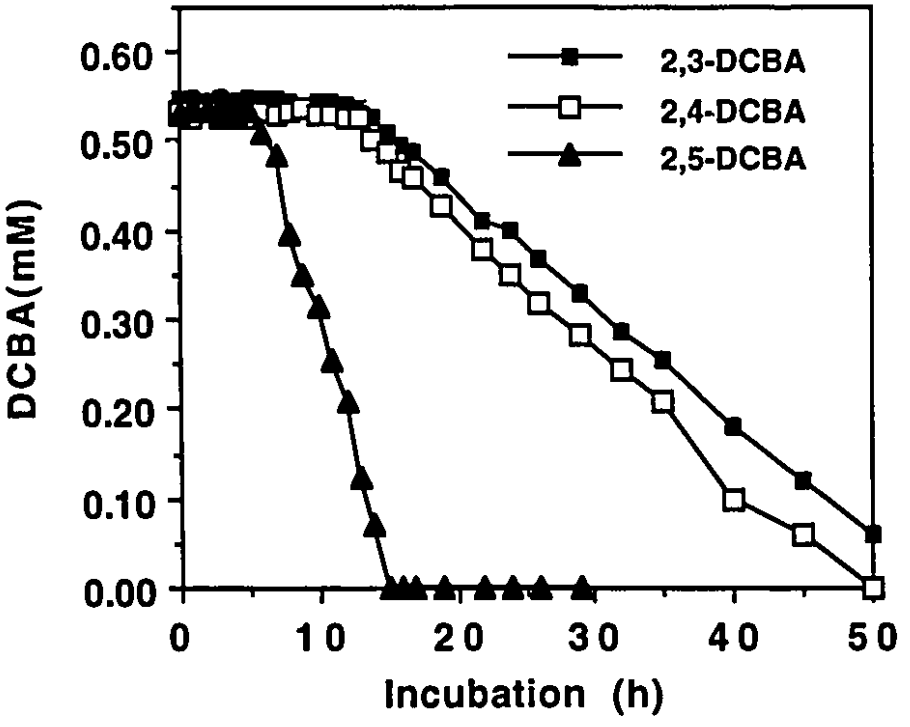

0.60
0.50
0.40
0.30
0.20
0.10
0.00
DCBA (mM)
0
10
20
30
40
50
Incubation (h)
2,3-DCBA
2,4-DCBA

Figure 6.5

Utilizatien of 2,3-DCBA, 2,4-DCBA , and 2,5-DCBA present in the growth medium simultaneously at the indicated concentrations by BRI 6011. For assay conditions see Materials and Methods.


2,3-DCBA
2,4-DCBA
2,5-DCBA
DCBA(mM)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
Incubation (h)

6.5 Discussion

Normally, degradation of chlorobenzoic acids appears to be favoured by chlorine in the 3 (*meta*) position and hindered by chlorine in the 2 (*ortho*) position, which reflects the regiospecificity of the benzoic acid 1,2-dioxygenase (Reineke and Knackmuss, 1978a; Parsons et al. 1988). However, dechlorination of the *ortho*-chlorine by 1,2-dioxygenation (Fetzner et al. 1992; Sylvestre et al. 1989; Engesser et al. 1989; Hickey and Focht 1990; Hernandez et al. 1991) and by reductive (van den Tweel et al. 1987) and hydrolytic dehalogenation (Higson and Focht 1990), has extended the potential number of CBAs degradable by axenic cultures. In all documented cases, aerobic degradation of 2-CBA appears to be via dioxygenation resulting in the formation of catechol (Fetzner et al. 1989a, 1989b, 1992; Engesser and Shulte 1989; Zaitsev and Karasevich 1984; Sylvestre et al. 1989) or in 3-CC (Hartmann et al. 1989; Sylvestre et al. 1989; Hickey and Focht 1990). Dioxygenation of *ortho*-substituted chlorobenzoic acids by 2-halobenzoate 1,2-dioxygenase forms a highly unstable compound, 2-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid when 2-CBA is the substrate, or a chlorinated form of this dihydrodiol when *ortho*-substituted dichlorobenzoic acids are the substrates, which spontaneously release carbon dioxide and the *ortho*-chloride forming either catechol or mono-chlorocatechol. Therefore, no dehydrogenase activity is required to yield the aromatic product, catechol (Fetzner et al. 1992). Several lines of evidence suggest that degradation of 2-CBA by BRI 3010 and BRI 6011 might be initiated by a 1,2-dioxygenation event, catalyzed by an enzyme with increased affinity for CBAs. BRI 3010 and BRI 6011 did not utilize CBAs anaerobically even in the presence of an alternate electron acceptor, nitrate. Therefore, the mechanism of reductive dechlorination described for 2,4-DCBA degrading organisms (van den Tweel et al. 1987; Zaitsev and Karasevich

1985), and hydrolytic dechlorination observed in 4-CBA degrading organisms (van den Tweel et al. 1987; Zaitsev and Karasevich 1985), is not expected to occur in BRI 3010 and BRI 6011. Oxygen uptake experiments indicated that growth of BRI 3010 and BRI 6011 on CBAs induced a second dioxygenase with increased CBA activity without a concomitant increase in activity on benzoic acid. Growth of both organisms on 2-CBA resulted in the stoichiometric release of chloride ions and the induction of catechol 1,2-dioxygenase instead of chlorocatechol 1,2-dioxygenase, shown here and in a previous study (Miguez et al. 1990), suggesting that dehalogenation might have occurred prior to the cleavage of the aromatic ring. Interestingly, BRI 3010 and BRI 6011 grown on benzoic acid, oxidized chlorobenzoic acids and in particular 2-CBA at significant rates, suggesting that the benzoic acid-induced benzoic acid dioxygenase possesses relatively broad substrate specificity.

The enzyme involved in the dioxygenation of 2-CBA, possibly a 2-chlorobenzoic acid 1,2-dioxygenase, appears to also catalyze the oxygenation of the DCBAs utilized by both BRI 3010 and BRI 6011. The oxygen uptake profile of CBAs by BRI 3010 cells grown on 2,5-DCBA, and by BRI 6011 cells grown on 2,4- and 2,5-DCBA, is similar to that of 2-CBA grown cells. In contrast to 2-CBA-grown cells, growth on DCBAs induced a chlorocatechol 1,2-dioxygenase required for the cleavage of the chlorocatechol intermediates. It has been previously established (Miguez et al. 1990) that the induction and activity of the chlorocatechol 1,2-dioxygenase in BRI 3010 and BRI 6011 were similar. Furthermore, the membranes of both organisms were very permeable to lipophilic weak acids, including 2,4-DCBA (Section 5 of this thesis). Therefore, the absence of 2,4-DCBA metabolism by BRI 3010 suggests that this compound in not a substrate for the 2-chlorobenzoic acid dioxygenase. Indeed, the enzymes involved in the catalysis of 1,2-dioxygenation of *ortho*-substituted

chlorobenzoic acids in other organisms, have been shown to possess distinct and varied substrate specifities. In *P. putida* P111, degradation of 2- , 3- , 4-CBA, 2,3- , 2,4- , 2,5- , 3,5-DCBA, and 2,3,5-TCBA is initiated by 1,2-dioxygenation (Hernandez et al. 1991). Whereas, the 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS exhibited much narrower CBA substrate specificity (Fetzner et al. 1992). Attempts to determine benzoic acid dioxygenase activity with crude cell lysates of BRI 6011 were made; however, as reported by others (Engesser et al. 1990; Fetzner et al. 1992), the high endogenous NADH consumption and oxygen uptake by the lysate prevented determining the definitive mechanism of the initial substrate dioxygenation. This goal may be achieved by the purification of the benzoic acid and chlorobenzoic acid dioxygenases and/or the recovery and identification of metabolites from the degradation of benzoic acids and CBAs by resting cells of BRI 3010 and BRI 6011.

The increased rates of salicylic acid (2-HBA) and gentisic acid (2,5-DHBA) oxidation by BRI 6011 grown on benzoic acid is fascinating, suggesting the induction of the salicylic acid degradation pathway via gentisic acid as reported for *Pseudomonas* sp. JS150 grown on either naphthalene or salicylic acid (Haigler et al. 1992). However, in *Pseudomonas* sp. JS150, growth on benzoic acid did not induce the oxidation of salicylic acid. Furthermore, the cleavage of the aromatic ring in the salicylic acid pathway is normally via catechol 2,3-dioxygenase (*meta*-cleavage) (Chakrabarty 1972; Haigler et al. 1992), an enzyme which is not induced in BRI 6011. Precedence for the metabolism of salicylic acid by the *ortho*-pathway in *Pseudomonas* P_G, does exist (Williams et al. 1975).

The metabolic responses of a microorganism to a single substrate (ie. rates of uptake, oxygenation, and degradation), may not be entirely

representative of *in situ* microbial responses where complex substrate mixtures, varying in concentration and degradability, may be encountered. In a previous study (Miguez et al. 1990), the mixed substrate utilization of 2,4- and 2,5-DCBA was shown to be sequential as defined by Harder and Dijkhuizen (1982), where utilization of a metabolizable substrate (2,4-DCBA) is prevented by the presence of a second metabolizable substrate (2,5-DCBA) and is only metabolized upon removal of the latter substrate. Metabolism of 2,4-DCBA by the culture was immediately halted upon the addition of 2,5-DCBA which in turn was rapidly metabolized (Miguez et al. 1990). This sequential pattern of substrate utilization may be a reflection of an early enzymatic reaction in the degradation pathway with greater affinity for 2,5-DCBA. Similarly, when 2-CBA was included in the growth medium with 2,4- or 2,5-DCBA, utilization of the dichlorobenzoic acid did not occur until most of the 2-CBA was depleted from the growth medium. The simultaneous (non-sequential) utilization pattern of 2,3- and 2,4-DCBA may reflect the action of an enzyme with similar affinity for both substrates. Uptake of these substrates by BRI 3010 and BRI 6011 is by simple diffusion and is therefore not expected to influence the substrate utilization sequence observed (Miguez et al. 1993). Assuming 1,2-dioxygenation and *ortho*-dechlorination, 3- and 4-CC are the expected metabolites of 2,3-DCBA, and of both 2,4- and 2,5-DCBA degradation, respectively. Since the turnover rates of these catechols were previously shown to be similar (Miguez et al. 1993), the cleavage event of the aromatic ring is not expected to have an influence on the substrate preference observed. These results suggest that the dioxygenation of the chlorinated benzoic acids in BRI 6011 is catalysed by the same enzyme. In an environment where 2-CBA or 2,5-DCBA are the predominating substrates, or where a continuous input of these two substrates occurs, the ability of BRI 6011 to utilize and remove 2,3-

and 2,4-DCBA would be compromised. Simultaneous utilization of the closely related aromatic compounds, chlorobenzene and toluene, by a natural isolate has recently been described (Pettigrew et al. 1991). However, the rate of utilization of either substrate when individually present in the growth medium was greater than when the substrates were mixed. Therefore, determining the utilization rate of individual aromatic substrates in single substrate systems, may not adequately portray the utilization of these substrates when mixed.

Section 7

General Conclusions

Two strains of *Alcaligenes denitrificans*, designated BRI 3010 and BRI 6011 were isolated from an environmental soil sample contaminated with PCB by enrichment culture using 2,5-DCBA and 2,4-DCBA, respectively, as sole sources of carbon. Strains BRI 3010 and BRI 6011 could metabolize benzoic acid, 2-CBA, 2,3- , and 2,5-DCBA. In addition strain BRI 6011 could also degrade 2,4-DCBA. The propensity for the formation of *ortho*-substituted chlorinated benzoic acids as a result of microbial co-metabolism of PCBs and the notorious recalcitrance of these chlorinated benzoic acid metabolites to pursuant microbial attack, render these two isolates extremely important players in the overall degradation of PCBs in nature. Consequently, BRI 3010 and BRI 6011 were physiologically characterized.

Cleavage of the aromatic ring is a crucial step whereby recalcitrant molecules are converted to metabolites which can enter the cell's central metabolic pathways. BRI 3010 and BRI 6011 possess two different *ortho*-cleaving enzymes, a catechol 1,2-dioxygenase and a chlorocatechol 1,2-dioxygenase. The latter is crucial for the dissimilation of chlorinated catechols and is induced by growth in the presence CBAs, with the exception of 2-CBA. *Meta*-cleavage activity (catechol 2,3-dioxygenase) was not detected in cells grown on benzoic acid or CBAs and therefore was not a factor in the degradation of these substrates. The purification and characterization of the chlorocatechol 1,2-dioxygenase from BRI 6011 revealed high affinities for 3- , 4-CC, 3,5- , and 3,6-DCC. These are all potential metabolites of the degradation of CBAs utilized by the organism and therefore indicate possession of a well adapted system. Interestingly, a chlorocatechol 1,2-dioxygenase isolated from

Pseudomonas sp B13 was very similar, on the basis of biochemical and genetic characterization, to the purified enzyme from BRI 6011. *Pseudomonas* sp. B13 is capable of degrading only 3-CBA, a substrate not utilized by either BRI 3010 and BRI 6011, although the expected intermediates, 3-, and 4-CC, are effectively metabolized. The increased versatility for CBA degradation by BRI 3010 and BRI 6011 can therefore not be attributed to a more specialized chlorocatechol 1,2-dioxygenase. Consequently, the dioxygenation of the CBAs and the uptake of these substrates by BRI 3010 and BRI 6011 was investigated to determine the factor(s) responsible for the apparent enhanced versatility.

The profile of oxygen uptake in the presence of benzoic acid or CBAs by whole cells grown on benzoic acid or CBAs strongly suggested the existence of two benzoic acid dioxygenases, one with affinity for benzoic acid and another with affinity for CBAs. Several lines of evidence suggested that the latter enzyme may be classified as a 2-chlorobenzoic acid 1,2-dioxygenase. Growth on 2-CBA resulted in an increase in the rates of CBA oxidation without a concomitant increase in benzoic acid oxidation. Growth on 2-CBA did not induce chlorocatechol 1,2-dioxygenase activity. The expected catecholic intermediate from 2-CBA dioxygenation is catechol or 3-CC. Productive metabolism of 3-CC requires the presence of a chlorocatechol 1,2-dioxygenase. Hence, the efficient utilization of 2-CBA by BRI 3010 and BRI 6011 in the absence of chlorocatechol 1,2-dioxygenase activity, suggests the possibility that a 2-chlorobenzoic acid 1,2-dioxygenase is operative in these strains, converting 2-CBA to 2-chloro-3,5-cyclohexadiene-1,?-diol-1-carboxylic acid which spontaneously releases carbon dioxide and the *ortho*-substituted chlorine forming catechol. The sequential and marked preference of 2-CBA utilization over 2,4- and 2,5-DCBA, also suggests that the 2-chlorobenzoic acid dioxygenase may recognize and dioxygenate 2,4- and 2,5-DCBA, albeit with

diminished affinity. Dioxygenation of 2,4- and 2,5-DCBA by this enzyme would yield 4-CC, which is efficiently metabolized by the organism. Dioxygenation of 2,3-DCBA by this enzyme would yield 3-CC, which is also efficiently metabolized.

Uptake of benzoic acid by BRI 3010 and BRI 6011 was inducible, exhibited saturation kinetics, and the substrate was accumulated against a concentration gradient by a factor of 8 and 10, respectively. In contrast, the uptake of 2,4-DCBA into BRI 6011 showed no evidence of saturation kinetics nor was there intracellular accumulation against a concentration gradient suggesting simple diffusion of the substrate across the cytoplasmic membrane down a concentration gradient in response to an inside alkaline environment. The strong inhibition of benzoic acid uptake by a wide range of CBAs and other structurally related compounds and the lack of observed competitive inhibition of benzoic acid by the analogues tested, revealed that the membranes of BRI 3010 and BRI 6011 are very permeable to lipophilic weak acids. In contrast, the membrane of *Pseudomonas* sp. B13 was much less penetrable. Benzoic acid uptake into *Pseudomonas* sp. B13 was inhibited by benzoic acid and 3-CBA only. The fact that 3-CBA apparently diffused into BRI 3010 and BRI 6011 but was not metabolized is consistent with the inability of the benzoic acid 1,2-dioxygenase or the chlorobenzoic acid 1,2-dioxygenase to recognize 3-CBA as a substrate. Similarly, since 2,4-DCBA apparently diffuses across the membrane and the expected catecholic intermediates of 2,4-DCBA metabolism are metabolizable by BRI 3010, this suggests that the major difference between BRI 3010 and BRI 6011 may be the inability of the 2-chlorobenzoic acid 1,2-dioxygenase in BRI 3010 to recognize 2,4-DCBA as a substrate. In section 3.5 of this thesis, an arguement for the possession of two different uptake systems

in BRI 6011 with different affinities for 2,5-DCBA was made. However, uptake studies using radio labeled substrates do not corroborate these arguments.

The sequential utilization pattern of CBAs observed in BRI 6011 exemplifies the importance of understanding an organism's response to mixed substrate growth conditions, in order to be able to optimize and predict the bioremediation efficacy of xenobiotic contaminated environments. Although BRI 6011 is metabolically versatile with respect to CBAs, the presence of a preferred CBA isomer substrate will retard the utilization of other metabolizable CBAs. Since environments are seldom contaminated by single chemical species, this type of sequential utilization is quite likely but not well known. Also, the uncoupling of metabolism by both metabolizable and non-metabolizable aromatic acids when present at sufficiently high concentrations, can be a potential constraint to bioremediation, a scenario which is quite likely in heavily contaminated environments.

The work presented here has raised several potentially fruitful research avenues to pursue. The recovery and identification of metabolites from the degradation of CBAs should be done in order to confirm the dioxygenation mechanism of *ortho*-substituted mono- and dichlorobenzoic acids. Isolation and purification of the benzoic acid and chlorobenzoic acid dioxygenases from BRI 3010 and BRI 6011 would with certainty ascertain the difference between BRI 3010 and BRI 6011 with respect to 2,4-DCBA utilization. This might also reveal the step involved in the metabolism of CBAs which differentiates *Pseudomonas* sp. B13 and *A. denitrificans*, strains BRI 3010 and BRI 6011. Permease mediated transport of benzoic acid, hydroxybenzoic acids, and 4-CBA have been described in the literature. However, to my knowledge, these proteins have not been isolated and characterized. Furthermore, although the operonic structure and location of the genes encoding the benzoic acid

degradation pathways have been established, the identification and location of the genes encoding the benzoic acid permeases are not known. Determining their genetic location would reveal whether or not they are part of the benzoic acid operon and consequently the mechanism by which their expression is regulated.

Finally, the use of BRI 6011 in co-culture with efficient CBP degraders should be tested to ascertain whether this combination enhances degradation of chlorinated biphenyls.

Section 8

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