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CHARACTERIZATION OF MICROBIAL POPULATIONS
IN ANAEROBIC FOOD WEBS THAT REDUCTIVELY
DECHLORINATE CHLOROPHENOLS

presented by

Robert Allan Sanford

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CHARACTERIZATION OF MICROBIAL POPULATIONS IN ANAEROBIC FOOD WEBS THAT REDUCTIVELY DECHLORINATE CHLOROPHENOLS

By

Robert Allan Sanford

A DISSERTATION

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1996

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ABSTRACT

CHARACTERIZATION OF MICROBIAL POPULATIONS IN ANAEROBIC FOOD WEBS THAT REDUCTIVELY DECHLORINATE CHLOROPHENOLS

By

Robert Allan Sanford

In this study the anaerobic dechlorination activities of mono- and dichlorophenols (CPs and DCPs) in nitrate-fed microcosms were characterized. Denitrification was shown to not be coupled to the reductive dechlorination of CPs or DCPs. Enrichment cultures derived from the microcosms exhibited different substrate specificity for individual CP or DCP isomers depending on the substrate fed to the enrichment. Four dechlorinating cultures with different dechlorination patterns were studied further, with two new halorespiring microbial isolates and two novel meta-dechlorinating enrichment cultures characterized. The first isolates, strains 2CP-C and 2CP-3, were identified as *Anaeromyxo dehalogenans*, the first anaerobic myxobacteria characterized. Strains 2CP-C and 2CP-3 are able to grow using acetate as an electron donor and 2-CP as an electron acceptor. The second enrichment culture exhibited the ortho-dechlorination of 2,3-DCP and yielded the new halorespiring isolate *Desulfitobacterium chlororespiricans* strain Co23. Strain Co23 coupled the partial oxidation of lactate to reductive dechlorination to gain energy for growth. The third culture exhibited meta-dechlorination of 3-CP under methanogenic conditions. Inhibitors for methanogenesis and eubacterial growth added to the culture indicated that a eubacterial population was responsible for dechlorination. The final enrichment culture derived from the microcosms dechlorinated ortho- and meta- substituted chlorines from 2,5-DCP, 2- plus 3-CP simultaneously. 62% of the available reducing equivalents were used for

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reductive dechlorination, indicating that this was likely to be a respiratory process. H_2 was observed in the headspace of the microcosm used for the OM enrichment culture, which indicated that H_2 was a very important intermediate in this anaerobic ecosystem. The microbial population responsible for H_2 production from formate in the OM enrichment culture was isolated and characterized. Strain FOX1 is a novel sulfate-reducing bacterium with the novel ability to grow on formate oxidation to H_2 and CO_2 as the sole source of energy. Energetically FOX1 is able to grow at the lowest $\Delta G'$ observed for any microorganism and produces H_2 at greater rates and to higher concentrations than previously reported.

To my father Allan and in memory of my grandfather Roscoe

both who's passion and dedication to science inspired me.

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Chapter I

INTRODUCTION

Overview

During the mineralization of xenobiotic compounds, microbial communities require a terminal electron acceptor in order to grow and capture energy from catabolic reactions. In addition to oxygen; nitrate, ferric iron, halogenated organic compounds, sulfate, carbon dioxide and other organic compounds have been shown to act as electron acceptors for growth in bacteria. In the environment microorganisms utilize these electron acceptors in accordance with their relative abundance and the energy available from their reduction (Lovley and Goodwin 1988; Lovley et al. 1994). Although chlorophenolic compounds are common contaminants and their degradation under aerobic, methanogenic and sulfidogenic conditions has been well studied, only a few studies have investigated dechlorination and degradation under denitrifying conditions (Genthner et al. 1989; Häggblom et al. 1993). Since it has been shown that nitrate can act as a terminal electron acceptor during growth on xenobiotic compounds under anoxic conditions (Braun and Gibson 1984; Bossert et al. 1986; Hutchins et al. 1991; Kaiser and Bollag 1991; Häggblom et al. 1993), it would be reasonable to look for denitrifiers able to degrade chlorophenols. Since reductive dechlorination of chlorophenols is energy yielding under anoxic conditions,it may provide a benefit to a particular population of denitrifiers or to the microbial community.

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The objectives of this study were rooted in the goals established for the former Novel organisms thrust group within the Center for Microbial Ecology. The primary questions that this study addressed are as follows:

- Do denitrifying microbial populations exist that can dechlorinate and mineralize chlorophenols?
- What are the microbial populations responsible for reductive dechlorination of chlorophenols?
- Of what energetic value is the dechlorination activity to the microbial populations within the chlorophenol degrading anaerobic community?
- What is the influence of electron donors on the flux of energy within the anaerobic food-web? Particularly what is the energetic relationship between formate and H_2?

To study the potential for dechlorination under anoxic conditions in the presence of nitrate, I set up microcosms containing different soils. Microcosms received low concentrations of nitrate and mixtures of monochlorophenols or dichlorophenols. Both dechlorination and denitrification were observed in these microcosms, with complete degradation of 2-chlorophenol (CP), 3-CP, 2,3-dichlorophenol (DCP), and 2,5-DCP occurring. My results from enrichment culture studies indicated a complex anaerobic food-web was operating in the microcosms, with at least five different dechlorinating populations, and two pathways of phenol degradation: methanogenic and denitrifying. I then focused on characterizing the different dechlorinating populations and analyzing the energy flow in this anaerobic food web.

I chose four different dechlorinating enrichment cultures for further study. From these cultures I isolated and characterized two new microorganisms capable of coupling growth to reductive dechlorination, referred to as halorespiration. These consisted of two new strains of the 2-CP halorespiring *Anaeromyxo dehalogenans* , the first anaerobic Myxobacteria, and a novel spore forming microorganism, named *Desulfitobacterium halorespiricans*, that reductively dechlorinated 2,3-DCP and 3-Cl-4-hydroxybenzoate. A third

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dechlorinating culture I studied was an enrichment that exhibited meta-dechlorination of 3-CP under methanogenic conditions. Experiments were completed to evaluate what electron donors were important for dechlorination and if the methanogen population was important for this activity. The fourth dechlorinating enrichment culture I selected yielded no isolates but exhibited evidence of halorespiration activity. This OM (for *ortho-* and *meta-*dechlorinating) enrichment culture had the novel ability to remove both chlorines from 2,5-DCP simultaneously while using H_2 and CO_2 as the sole source of energy and carbon. Formate could also serve as an electron donor, but was stoichiometrically converted to H_2 before the onset of reductive dechlorination. My efforts to cultivate the dechlorinating culture on solid medium resulted in the isolation of a novel H_2 producing microorganism FOX1.

Since H_2 was potentially an important intermediate in the anaerobic food-web containing the dechlorinating populations, I evaluated the role microbes like the formate-oxidizing FOX1 might have in the anaerobic ecosystem. I determined that FOX1 was actually able to grow on formate alone with acetate added for incorporation into biomass. Formate oxidation coupled to the production of H_2 was the sole source of energy for this microorganism. FOX1 was the first organism for which this type of growth has been demonstrated. An analysis of the free-energy associated with this process revealed two other novel features, FOX1 grew at the lowest $\Delta G'$ ever observed for a microorganism and it catalyzed this reaction to thermodynamic equilibrium. The existence of microbial populations like FOX1 suggests a more energetically dynamic role for formate cycling in anaerobic ecosystems.

Background

Biodegradation Under Denitrifying Conditions. Biodegrading denitrifiers are underinvestigated relative to their potential practical importance

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in bioremediation. Nitrate is potentially an attractive alternative to oxygen because of its solubility, mobility, e^- accepting capacity, and low toxicity in the environment. Recent field studies support this strategy and have yielded evidence that nitrate addition stimulated removal of BTEX from contaminated groundwater (Hutchins et al. 1991).

Traditionally, the degradation of xenobiotic compounds, chlorinated and or aromatic, has been evaluated under aerobic or strictly low redox conditions. Much of the aerobic microbial research has focused on oxygen requiring oxygenases involved in aromatic or methane oxidative pathways as well as on co-metabolism of chlorinated solvents like trichloroethene (TCE) and dichloroethene (DCE) (Chaudry and Chapalamadugu 1991; Hardman 1991). Anaerobic biodegradation research has primarily focused on reductive dechlorination of highly substituted halogenated organic compounds, as observed in methanogenic and sulfidogenic enrichments (Suflita et al. 1982; Reineke and Knackmuss 1988; Hardman 1991; Mohn and Tiedje 1992). As a result the prevailing scientific opinion had been that oxygenases were required for aromatic ring cleavage and that highly chlorinated chemicals only were transformed, but not mineralized, under strictly anaerobic conditions. Recently, studies have identified denitrifying and sulfate-reducing populations that can mediate the anaerobic oxidation of toluene (Evans et al. 1991; Beller et al. 1992; Fries et al. 1994). This work has encouraged further research that focused on the degradation potential of denitrifiers and the moderate redox conditions in which they thrive.

Chlorinated aromatic compounds have properties that must be considered when considering their degradability under denitrifying conditions. The major one is the degree of chlorination and its impact on the catabolic pathway. For example, can a moderately chlorinated (2-3 Cl) aromatic

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compound be degraded via a pathway similar to the toluene degradation pathway under denitrifying conditions? If this is possible, then these compounds could be degraded by pure cultures under denitrifying conditions, as they could presumably follow the dechlorination steps used by non-oxygen requiring reactions found in aerobic or anaerobic pathways (Reineke and Knackmuss 1988; Chaudry and Chapalamadugu 1991; Hardman 1991; Mohn and Tiedje 1992).

Phenolic compounds, especially chlorinated ones, have been used extensively in industry and therefore have become common environmental pollutants. Most are relatively soluble under the right conditions and have low volatility. This makes them very mobile in aqueous environments and a potential source of groundwater pollution. Some of the early work on biodegradation under denitrifying conditions has focused on similar, but non-halogenated phenolic compounds, especially cresols and phenol (Häggblom et al. 1990; Dangel et al. 1991; Rudolphi et al. 1991; Seyfried et al. 1991). It is reasonable to predict that degradation pathways of chlorinated phenolic compounds may be related to those previously identified in similar non-halogenated compounds.

Naturally occurring halogenated compounds. Although much research has focused on the biodegradation of xenobiotic chlorinated organic compounds, there has been little awareness of the existence of natural halogenated compounds. It is the naturally biogenic or geogenic halophenolic compounds that are probably responsible for the microbial mediated catabolic activities that are utilized today for the bioremediation of xenobiotic analogs to those compounds. In a recent review Gribble (1992) described hundreds of naturally occurring halogenated compounds. Some of these are halogenated phenolic compounds, analogous to the chlorophenols that have been used for

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extensive aerobic and anaerobic biodegradation research (Figure 1.1). These natural compounds are sometimes present in fairly high concentrations in the environment. For example, a white-rot fungus was shown to produce up to 70 mg kg^{-1} chlorinated anisyl metabolites (e.g. 3-chloroanisaldehyde) in soil litter (de Jong et al. 1994). Compounds shown in Figure 1.1 are found in very diverse environments, from the sex pheromone of the lone star tick to the bromophenolic excretions of marine tube worms. Chlorinated and brominated tyrosine has even been shown to be incorporated into certain proteins in mollusks(Gribble 1992). The ubiquitous nature of these compounds leads to the reasonable hypothesis that considerable microbial diversity would have evolved the ability to degrade these compounds under anaerobic and aerobic conditions. This has been demonstrated with bromophenol-producing marine organisms, from which anaerobic degradation has been observed and a pure culture has been isolated (King 1988; Steward et al. 1995).

Reductive dehalogenation of chlorophenols. Reductive dehalogenation of chloroaromatic compounds has been well studied in anaerobic ecosystems (Suflita et al. 1982; Mohn and Tiedje 1992; Nicholson et al. 1992). As an example, the dechlorination pathways for four trichlorophenol isomers and six dichlorophenol isomers are shown in Figure 1.2. It is evident that anaerobically these substrates have been observed to be dechlorinated completely to phenol and 4-CP. Several of the chlorophenols have been shown to serve as terminal electron acceptors in microbial cultures, illustrated as heavy arrows in Figure 1.2 (Cole et al. 1994; Utkin et al. 1994) (this thesis). Even those reactions that appear to support higher cell yields, have been shown to cometabolically dechlorinate more complex chlorophenols (Utkin et al. 1995). For example *Desulfitobacterium dehalogenans* strain JW/IU DC1 was selected in enrichment cultures that exhibited ortho-dechlorination of 2,4-

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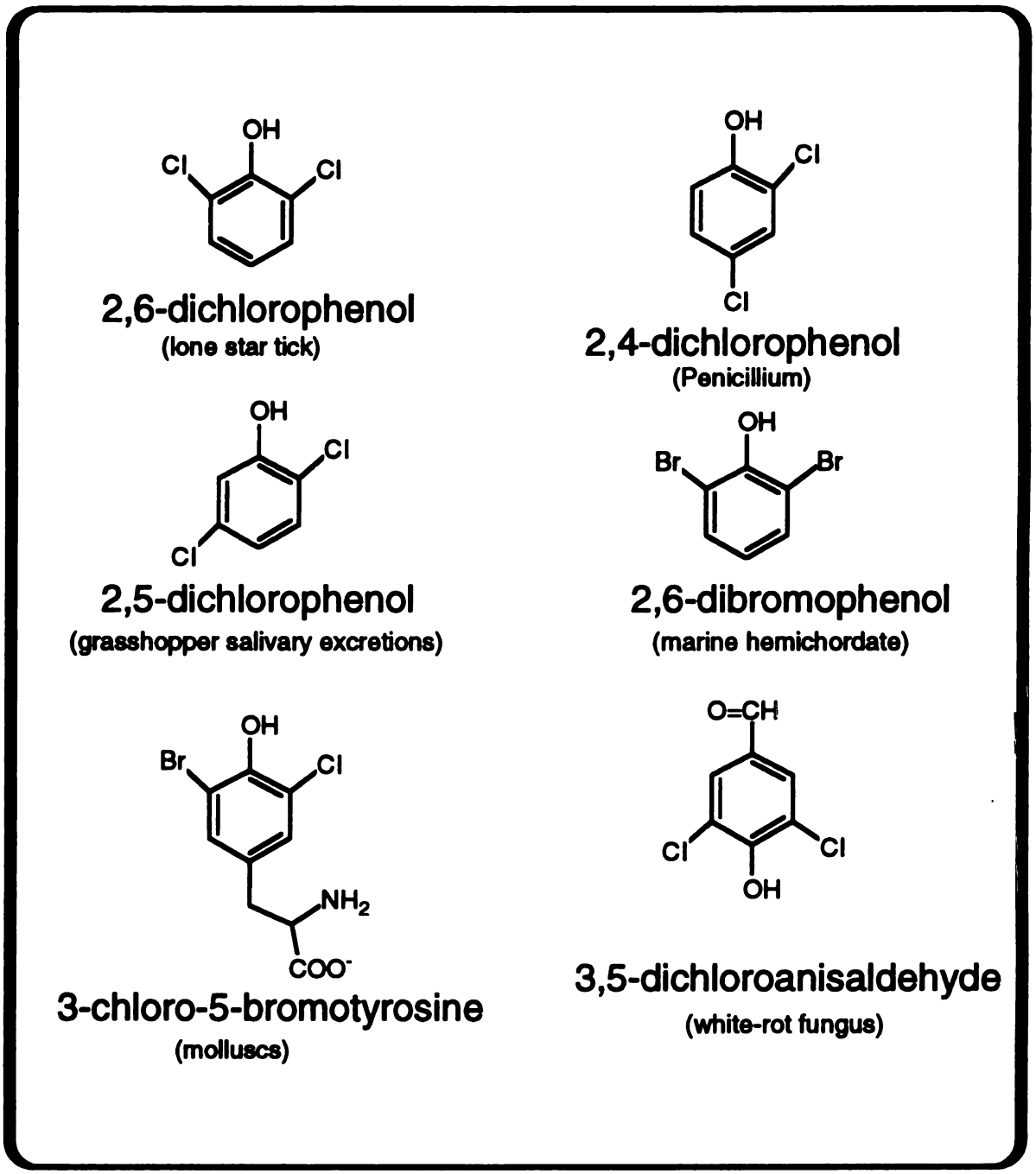

Figure 1.1. Several naturally occurring halogenated phenolic compounds. Taken from Gribble (1992) and de Jong (1994).

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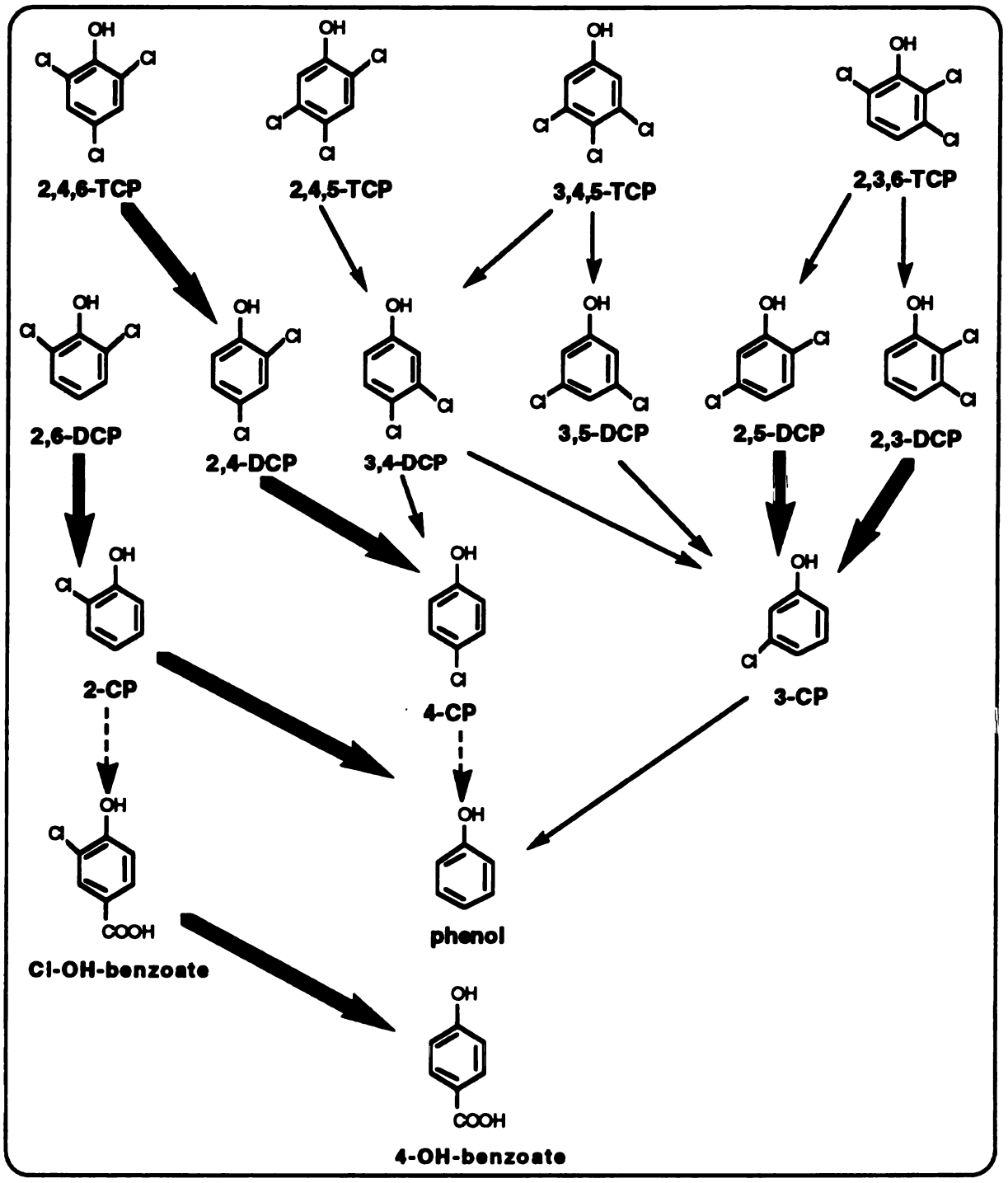

Figure 1.2. Anaerobic degradation pathways observed for trichlorophenols. Heavy arrows denote reductive dechlorination reactions known to support growth via halorespiration. Arrows with dotted lines indicate transformations that are likely to occur and arrows with thin-lines indicate reactions that have been observed in anaerobic systems.

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DCP, and it has been shown that when induced this strain can also dechlorinate pentachlorophenol (PCP) to 3,4,5-TCP. In general ortho reductive dechlorination of chlorophenols is the most common reaction observed followed by meta- and para-dechlorination (Nicholson et al. 1992).

The free-energy, ΔG, associated with reductive dechlorination of chloroaromatic compounds is significantly exergonic (Dolfing and Harrison 1992), very close to the $\Delta G^{o'}$ of nitrate reduction to nitrite (Table 1.1). A comparison of the $\Delta G^{o'}$ associated with various electron-accepting reactions are shown in Table 1.1. When coupled to the oxidation of H_2 as an electron donor it is evident that reductive dechlorination potentially provides more energy than fumarate reduction, sulfate reduction or methanogenesis. Halorespiration is the ability to utilize the energy from this reaction for growth. This process has only been found in a few microorganisms. *Desulfomonile tiedjei* strain DCB-1 was the first isolated bacterium shown to grow and couple ATP generation to the reductive dechlorination of 3-chlorobenzoate (3-CBA) (Dolfing 1990; Mohn and Tiedje 1990). More recently an anaerobic 2-CP halorespiring microbe phylogenetically related to the myxobacteria was isolated and characterized (Cole et al. 1994). *Desulfitobacterium dehalogenans* strain JW/IU DC1 exhibited increased cell-yields when it was dechlorinating 3-chloro-4-hydroxyphenylacetate with pyruvate as the electron donor (Utkin et al. 1994). Non-aromatic compounds such as tetrachloroethene (PCE) are also known to support halorespiration. For example, Holliger et al. (1993) described the anaerobic PCE dechlorinating microbe *Dehalobacter restrictus* that only grew when H_2 was provided as an electron donor and PCE was added as an electron acceptor (Holliger and Schumacher 1994). PCE was also utilized by *Dehalospirillum multivorans* as a terminal electron acceptor, however this strain exhibited a much broader range of substrates that would support growth

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$N_2O + 2H^+$

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$NO_3^- + 6H^+$

$NO_3^- + 2H^+$

$C_6H_4ClOH +$

$Fumarate^{-2} +$

$SO_4^{-2} + 9H^+$

$HCO_3^- + 8H^+$

$2HCO_3^- + 9H^+$

$2H^+ + 2e^-$

e- donating

$H_2 \longrightarrow 2H^+$

$HCOO^- + H_2C$

$CH_3CH_2CH_2CH_2CC$

Table 1.1 Relevant half-reactions for reductive dechlorination of monochlorophenol and other electron acceptors. Half-reactions for formate, H_2 and butyrate as electron donors are also shown. $\Delta G^{o'}$ values shown where known or calculated from theoretical thermodynamic calculations (Thauer et al. 1977; Criddle et al. 1991; Dolfing and Harrison 1992).

Half-reaction	$\Delta G^{o'}$ (KJ/mole e-)
e- accepting reactions:	
$N_2O + 2H^+ + 2e^- \longrightarrow N_2 + H_2O$	-131
$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$	-79
$NO_3^- + 6H^+ + 5e^- \longrightarrow 1/2\,N_2 + 3H_2O$	-72
$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$	-41.5
$C_6H_4ClOH + 2H^+ + 2e^- \longrightarrow C_6H_5OH + HCl$	**-40**
$Fumarate^{-2} + 2H^+ + 2e^- \longrightarrow succinate^{-2}$	-3
$SO_4^{-2} + 9H^+ + 8e^- \longrightarrow HS^- + 4H_2O$	+21
$HCO_3^- + 8H^+ + 8e^- \longrightarrow CH_4 + 3H_2O$	+23
$2HCO_3^- + 9H^+ + 8e^- \longrightarrow Acetate^- + 4H_2O$	+27
$2H^+ + 2e^- \longrightarrow H_2$	+40
e- donating reactions:	
$H_2 \longrightarrow 2H^+ + 2e^-$	-40
$HCOO^- + H_2O \longrightarrow HCO_3^- + 2H^+ + 2e^-$	-39.4
$CH_3CH_2CH_2COO^- + 2H_2O \longrightarrow 2\,CH_3COO^- + 5H^+ + 4e^-$	-28

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(Neumann et al. 1994; Neumann et al. 1995; Schlolz-Muramatsu et al. 1995). The halorespiring strains described represent a wide-range of phylogenetic diversity, suggesting that the ability to utilize halogenated compounds as respiratory electron acceptors has perhaps evolved many times.

Many of the dechlorination reactions of chlorophenols observed in anaerobic environments and enrichments do not appear to be linked to any energetic benefit to a particular microbial population. The dechlorination of polychlorinated biphenyls often has been associated with at least some methanogenic activity, where methane accounts for most of the reducing equivalents from electron donors consumed in these anaerobic cultures (Bedard and Quensen III 1995). Even though some evidence of methanogen mediated para-dechlorination of PCB congeners has been discovered, the predominant energy yielding reaction in most PCB enrichment cultures was not reductive dechlorination. One recent study showed that reductive dechlorination of chlorophenols continues in the absence of methanogenesis and dechlorination ceased when the eubacterial specific antibiotic vancomycin was added (Perkins et al. 1994). This suggested that a eubacterial population was responsible for dechlorination; however, even when methanogenesis was inhibited homo-acetogenesis became the major electron sink for the ecosystem. Since both methanogenesis and acetogenesis yield less free-energy than reductive dechlorination it is apparent that halorespiration is not likely to be occurring in these anaerobic enrichment cultures. If a halorespiring population was present, it should have a competitive advantage over the methanogens and acetogens present in the community and eventually would displace the other microbial populations. When this doesn't occur, alternative mechanisms should be considered. First it is possible that the reductive dechlorination is a fortuitous activity that is beneficial to the anaerobic food-web by serving as a H_2

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sink. For example, obligate H_2 generating syntrophs would benefit energetically if some of the excess reducing equivalents they produce were channeled toward the dechlorination of chlorophenols or other halogenated organic compounds instead of to H_2 synthesis. This is similar to halorespiration, except the energy from the reductive-dehalogenation reaction is not available to the microorganism. Alternatively reductive dechlorination could be a detoxification mechanism, however the specificity exhibited in enrichment cultures for these reactions and the persistence of some chlorinated isomers suggest that this might not be the case.

Energy flow in anaerobic food webs: the role of formate and H_2. The oxidation of small chain organic acids is largely mediated by syntrophic microorganisms in anaerobic environments. These reactions are exergonic only under low H_2 concentrations, thus a microbial population capable of consuming H_2 to low concentrations must be present in order for the syntroph to grow and survive (Schink and Friedrich 1994). For example the $\Delta G^{o'}$ for butyrate oxidation coupled to H^+ reduction to H_2 is + 12 kJ/ mole e^- (Table 1.1), therefore butyrate will only support growth if the $\Delta G'$ can be lowered by consuming the H_2 produced. Formate, however, is close to thermodynamic equilibrium with H_2 and it has been proposed that syntrophs may rely on formate as an electron carrier instead of H_2 (Thiele et al. 1988; Boone et al. 1989; Schink and Friedrich 1994). The arguments for formate as this electron carrier are that it is more soluble and has a higher diffusion coefficient than H_2, therefore energy will be dissipated more quickly from a formate producer than a H_2 producer. A major assumption to the energetic equivalency of these two potential electron carriers is that under physiological conditions formate and H_2 are in equilibrium. Anaerobic aquifers have been shown to have an obvious disequilibrium between formate and H_2 (McMahon and Chapelle 1991), with

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formate oxidation favored thermodynamically. In this type of environment it is not likely that formate is the primary electron carrier for syntrophic microorganisms.

Formate is a common intermediate in anaerobic degradation processes, and serves as a substrate for many terminal anaerobic processes such as acetogenesis, methanogenesis and sulfidogenesis. Although formate often is equated to H_2 in anaerobic microbial ecosystems (Stams 1994), there have been some interesting observations made in the past that suggest that these two compounds are not equivalent as energy substrates. In comparative experiments using formate or $H_2 + CO_2$ the yield of cells per mole of methane produced by *Methanobacterium formicicum* was found to be greater for formate (Schauer and Ferry 1980). The sulfate-reducer *Desulfovibrio vulgaris* was shown to have a higher cell-yield per mole SO_4^{-2} reduced when formate was the substrate instead of $H_2 + CO_2$ (Badziong and Thauer 1978; Magee et al. 1978). Hungate et al. (1970) suggested that formate was oxidized to H_2 and CO_2 by non-methanogenic microorganisms in the rumen where methanogens are one of the predominant groups of organisms present. In addition, it was suggested that H_2 and CO_2 were converted to methane and not formate in the rumen (Hungate 1967). All of these observations indicate that formate and H_2 do not behave equivalently in the environment, and that there may be energetic differences associated with their metabolism by microorganisms. Perhaps the most convincing evidence of the differences between formate and H_2 is alluded to in data presented by Stephenson and Stickland (1933). They report the conversion of formate into methane by a putative pure culture, however the same culture is reported to reduce sulfate to sulfide with H_2. Since there are no known methanogens that are also sulfidogens it is likely this was a coculture between a sulfate reducer and the methanogen. Formate would then support

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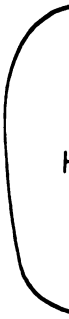
Sulfate reducers, particularly *Desulfovibrio* , are known to be able to readily metabolize both formate and H_2. This group of organisms has been shown to possess a least three types of hydrogenases, involved in either H_2 generation or consumption (Fauque et al. 1988; Widdel and Hansen 1992). Multiple hydrogenases have been identified in individual microorganisms, with both cytoplasmic and periplasmic locations within the cell. It has been proposed that cytoplasmic hydrogenases are involved in H_2 production, while periplasmic hydrogenases are involved in H_2 uptake (Widdel and Hansen 1992). This positioning of hydrogenases in *Desulfovibrios* is thought to be involved in hydrogen cycling which serves as a general mechanism for energy coupling in these microorganisms (Odom and Peck Jr. 1981). Hydrogen cycling facilitates the formation of a membrane potential by utilizing the diffusion of H_2 out of the cell as a proton transport mechanism. Hydrogenases in the periplasm can then oxidize the H_2 to protons and reduce membrane associated electron carriers. Electrons are then transported back across the membrane into the cell and are used to reduce the terminal electron acceptor, for example thiosulfate. A schematic of the enzyme localization and the electron flux across a theoretic sulfidogen membrane demonstrating hydrogen cycling is shown in Figure 1.3. Formate is shown as the electron donor. Since at least some sulfate reducers have a periplasmic location for their formate dehydrogenase, formate oxidation will give a net positive charge to the outside of the cell, thus enhancing the membrane potential (Widdel and Hansen 1992). In the presence of suitable electron donors, a considerable proton gradient can be established. If the cells are using formate and producing H_2, it is possible that H_2-cycling could still occur in the absence of electron acceptors, since high H_2 concentrations will



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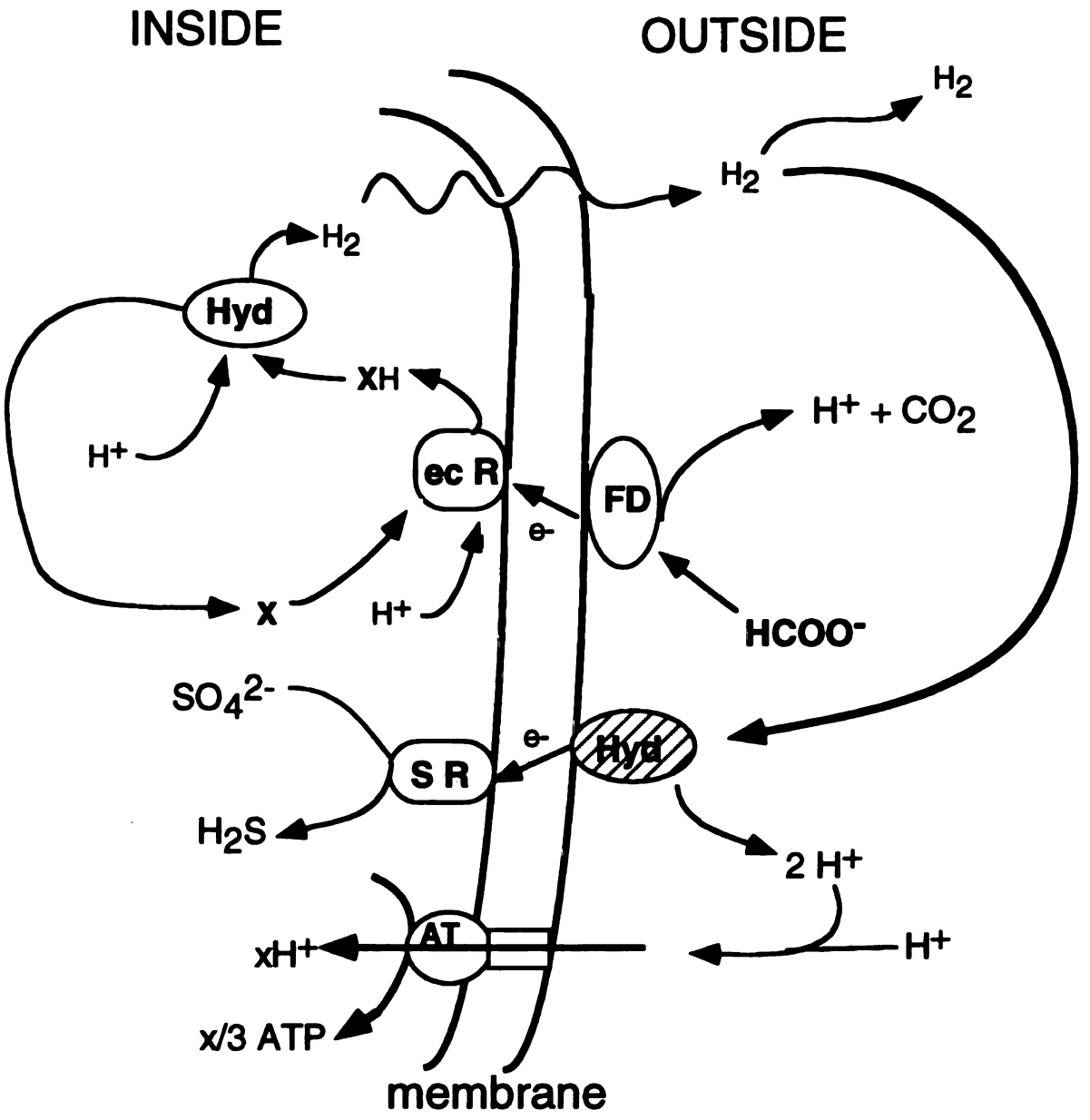

Figure 1.3. Schematic representation of H_2 cycling mechanism in a sulfate reducing microorganism using formate as an electron donor. FD = formate dehydrogenase; Hyd = hydrogenase; SR = sulfate reductases; ecR = electron carrier reductase and AT= ATP ase. Adapted from Odom and Peck (1981).

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not inhibit formate oxidation (Sanford, 1996, this dissertation). Therefore a proton gradient from formate oxidation can develop and even be enhanced with some proton generating hydrogenase activity outside the cell. One other sink for electrons besides the cytoplasmic hydrogenase, would be for the synthesis of biomass, which implies that growth could possibly occur from formate oxidation utilizing this type of mechanism.

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Chapter II

Chlorophenol dechlorination and subsequent degradation in nitrate-limited denitrifying microcosms

Abstract

The potential of using nitrate as a terminal electron acceptor to stimulate anaerobic degradation of mixtures of monochlorophenols (MCPs) or dichlorophenols (DCPs) was evaluated. Contaminated and non-contaminated soils were added to water-saturated anaerobic microcosms supplemented with 1 mM or 5 mM nitrate. Denitrification and dechlorination activities were present in three diverse soil types and were maintained upon refeeding both nitrate and the appropriate chlorophenol. However, dechlorination activity could only be serially transferred in enrichments with an added electron donor such as acetate. Dehalogenation activity in enrichments from four of the primary microcosms showed at least five different dechlorination reactions, each mediated by different microbial communities. Three of these are distinct *ortho*-dechlorinating paths; two are *meta*-dechlorinating and one is the para-dechlorination of 3,4-DCP. Simultaneous dechlorination and denitrification activities could be maintained in microcosms only in the presence of low nitrate concentrations. These processes were mediated by two separate microbial communities; one that dechlorinates without use of nitrate and one that denitrifies while oxidizing the aromatic ring. There was no evidence that dechlorination is mediated by the denitrifying community, however the maintenance of a denitrification potential using low (< 1 mM) nitrate concentrations may be useful for completing the food chain by stimulating the mineralization of phenol and benzoate.

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Introduction

Chlorophenolic compounds (CPs) have been used extensively in several industries and there has been considerable effort to study the degradation of these compounds under aerobic and strictly anaerobic conditions (Häggblom 1992; Mohn and Tiedje 1992). Several polychlorinated phenols and monochlorophenols (MCPs) are degraded under aerobic and anaerobic conditions (Boyd et al. 1983; Gibson and Suflita 1986; Hrudey et al. 1987; Genthner et al. 1989; Dietrich and Winter 1990; Hale et al. 1990; Zhang and Wiegel 1990; Madsen and Aamand 1992). The use of denitrifying conditions with chlorophenols as substrates has been relatively underinvestigated, and none of the previous studies have shown simultaneous dechlorination and nitrate reduction (Genthner et al. 1989; Häggblom et al. 1993). Relatively high nitrate concentrations were used in these studies, which may have inadvertently masked the potential of denitrifiers or dechlorinators acclimated to low nitrate concentrations . Nitrate is attractive as an electron acceptor because of its solubility and because denitrifying conditions promote complete mineralization of some aromatic compounds at reasonably rapid rates. Also, reductive dechlorination of chlorinated phenolic compounds, an exergonic reaction (Dolfing and Harrison 1992), would not be inhibited under low nitrate concentrations as it is by oxygen. Other studies have shown that dechlorination of MCPs does occur, presumably once nitrate has been depleted, but efforts to transfer these cultures to nitrate-containing media resulted in no dechlorination activity (Genthner et al. 1989). Nitrate-dependent chlorobenzoate-degrading enrichments, however, have been observed (Genthner et al. 1989b; Häggblom et al. 1993). This would indicate that there may be differences between the microbial communities that degrade these two types of compounds.

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Anaerobic chlorophenol dechlorination studies have predominantly used anaerobic sediments and sludges as inocula for enrichments or microcosms (Boyd et al. 1983; Gibson and Suflita 1986; Genthner et al. 1989; Kohring et al. 1989; Hale et al. 1991; Hendriksen et al. 1992; Häggblom et al. 1993). In contrast, the best source of denitrifying populations may be surface soils where the presence of nitrate and the fluctuating oxygen status provides regular selection for denitrifiers. Soils high in litter residue may also harbor reservoirs of naturally occurring chlorinated aromatic compounds. Several MCPs and dichlorophenols (DCPs) are known to be produced naturally (Berger 1972; Siuda and Debernardis 1973; Gribble 1992) and white rot fungi are known to produce up to 75 mg/kg litter of chlorinated anisyl metabolites (CAMs) in nature(de Jong et al. 1994).

We evaluated the degradation of mixtures of MCPs and DCPs under low nitrate concentrations (< 5 mM) in microcosms seeded with contaminated and relatively pristine soils. Mixtures were used to model the complex nature of wastes that occur in contaminated material and to broaden the selective pressure for dechlorinating microorganisms. Enrichment cultures were developed from microcosms that exhibited both denitrification and dechlorination activity. Our objectives were to determine the relationship of nitrate and denitrifiers to the degradation activity and to evaluate the substrate range and specificity of the dechlorination reactions mediated by these enriched populations. We observed at least five different dechlorination reactions in these microcosms and subsequent enrichments indicated they were carried out by different populations.

Materials and Methods

Source of microcosm inocula. Soils were obtained from several sources. Material from contaminated sites was obtained from Remediation-

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Technology and Ecova Corp. in Seattle, Washington. Sediment sludge samples from a paper pulp mill effluent treatment system in Ontario, Canada were provided by Dr. Roberta Fulthorpe. Soil samples from the Kellogg Biological Station were obtained from agricultural plots being treated with different levels of 2,4-D. A compost soil, high in organic matter, was sampled at the base of a compost pile in Lansing, Michigan. Two tropical soil samples were collected in the lowland rainforest of equatorial Cameroon by Dr. Roland Weller.

Microcosms. Microcosms were started in 160 ml serum bottles with 100 ml of boiled degassed medium and approximately 10 g of soil or sludge. Glass beads were used as a negative control. One microcosm set received a mixture of *o*-MCP, *m*-MCP and *p*-MCP at 200 μM each. The other set was supplemented with 125 μM each of 2,3-DCP, 2,4-DCP, 2,5-DCP and 3,4-DCP. Those MCPs and DCPs degraded during incubation were replenished at a concentration of 125 μM. Nitrate (as KNO_3) was provided at 5 mM or 1 mM to the microcosms. Nitrate was replenished to initial levels once it was depleted. After several additions only 1-2 mM nitrate was added to active microcosms. Serum bottles were closed with Teflon lined butyl rubber stoppers and incubated at 30 ° C.

Medium formulation. The following mineral salts medium (Stevens et al. 1992) was used for all cultures: 2 mM potassium phosphate buffer (pH 7.2 - 7.5), and per liter $CaCl_2 \cdot 2H_2O$ (0.015 g), $MgCl_2 \cdot 6H_2O$ (0.02 g,) $FeSO_4 \cdot 7H_2O$ (0.007 g), and Na_2SO_4 (0.005 g). A trace metals solution was added to give the following final concentration per liter: $MnCl_2 \cdot 4H_2O$ (5 mg), H_3BO_3 (0.5 mg), $ZnCl_2$ (0.5 mg), $CoCl_2 \cdot 6H_2O$ (0.5 mg), $NiSO_4 \cdot 6H_2O$ (0.5 mg), $CuCl_2 \cdot 2H_2O$ (0.3 mg), and $NaMoO_4 \cdot 2H_2O$ (0.1 mg). NH_4Cl was added to a concentration of 8 mM and 10 mM $NaHCO_3$ was added to buffer the headspace which contained a N_2:CO_2 ratio of 95:5. A vitamin solution (Wolin et al. 1963) was provided after cooling.

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Enrichment cultures. Aliquots from microcosms were transferred to fresh medium in serum bottles or anaerobic culture tubes with butyl rubber stoppers for the purpose of enriching the dechlorinating and denitrifying activities. Enrichments received nitrate at concentrations of 0, 1 or 5 mM. A subset of those with and without nitrate received 1 mM acetate or a mixture of volatile fatty acids (formate 625 μM, succinate 125 μM, propionate 125 μM, and butyrate 125 μM) as electron donors. The media were reduced with 0.2 mM cysteine and 0.2-0.5 mM Na_2S. Resazurin was added as a redox indicator. Individual MCPs and DCPs were used as substrates at a concentration of 125 μM. Cultures were incubated at 30 ° C.

Chemical analysis. Nitrate and nitrite were analyzed by using a Whatman Partisil 10 SAX column connected to a Schimadzu HPLC. The eluent was 50 mM phosphate buffer (pH = 3.0) pumped at a rate of 1 ml/min. UV adsorption at 210 nm was used for detection. Samples from primary enrichments were diluted 1:100 in deionized H_2O prior to nitrate and nitrite analysis.

MCPs, DCPs and aromatic products of dechlorination were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was phosphoric acid (0.1 %) buffered methanol at 1.5 ml/min using a gradient from 48% to 55% methanol. A Hibar RP-18 (10 μm) column was used. Peaks were quantified at 218 nm on a UV multiwavelength detector. Samples (1 ml) from the enrichments were taken, made basic with 10 μl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 μm filters prior to HPLC analysis.

The headspaces of the microcosms and enrichments were analyzed for CH_4, N_2, H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity (TCD) detector. Headspace pressures were normalized to atmospheric by venting with a needle prior to

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removing 0.3 ml of gas for injection into the GC. N_2O was quantified on a Perkin Elmer 910 GC with a Porapak Q column and ^{63}Ni -electron capture (ECD) detector. Denitrification products were measured in an Ar headspace.

Chemicals. MCPs and DCPs were obtained from Aldrich Chemical Co. Phenol was purchased from Malinkrodt and benzoate from Sigma Chemical Co.

Results

Dechlorinating denitrifying microcosms. Only seven of the 30 microcosms had both dechlorination and denitrification activities after one year of incubation (Table 2.1). No activity was observed with glass beads in the abiotic control. Microcosms with MCPs exhibited either *ortho*-dechlorination alone or simultaneously with *meta*-dechlorination. In contrast, DCP microcosms showed considerable dechlorination from all positions, and this resulted in the degradation of all the DCPs. Extensive gas production was observed indicating complete reduction of nitrate to nitrogen gas (Table 2.1). This was confirmed by GC analysis of the headspace gases which showed increases of N_2 and N_2O after nitrate and nitrite depletion. Two sets of microcosms, Trop I and Trop II, were very sensitive to the nitrate concentration, with both dechlorination and denitrification occurring only when the nitrate concentration was 1 mM or lower (Table 2.1). With the 5 mM Trop I microcosm, nitrate was slowly depleted and dechlorination did not occur until nitrate had been removed.

The compost soil microcosm with 5 mM nitrate containing a mixture of *o*-, *m*-, and *p*-MCP exhibited specificity for *o*-MCP and was sensitive to high nitrate concentrations. This microcosm showed no evidence of *m*-MCP or *p*-MCP disappearance over a 200 day period (Figure 2.1A). Phenol was the predominant dechlorination product observed, but was rapidly removed in the presence of nitrate (Figure 2.1B). Benzoate was also detected at lower

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Table 2.1. Summary of dechlorinating and denitrifying microcosms that were active within a year of incubation. Microcosms received mixtures of MCPs and DCPs and nitrate at 5 mM or 1 mM.

Soil or sludge	Substrates	Denitrification[a]	Dechlorination[b]	Products
Compost	MCPs; NO_3^-(5)[c]	+DC	++(*o*-MCP)	Phenol, benzoate
	DCPs; NO_3^-(5)	+DC	++(all DCPs)	Phenol, benzoate, *m*-MCP, *p*-MCP
Trop I	MCPs; NO_3^-(1)	+DC	++(*o*-MCP)	Phenol
	MCPs; NO_3^-(5)	+D	+(*o*-MCP)	Phenol
Trop II	MCPs;NO_3^-(1)	+DC	++(*o*-MCP)	Phenol
O1C	MCPs; NO_3^-(5)	+D	++(*o*-MCP, *m*-MCP)	Phenol
	DCPs; NO_3^-(5)	+D	+(all DCPs)	Phenol, *m*-MCP, *p*-MCP

[a] +DC indicates denitrification occurred concurrently with dechlorination. +D indicates that denitrification occurred, and nitrate was depleted prior to any other activity. Denitrification was determined by measuring N_2 or N_2O in the microcosm headspace after NO_3^- and NO_2^- disappearance.

[b] + or - indicates presence of dechlorination acitivity. Substrates dechlorinated are indicated in parentheses. ++ indicates a relatively higher level of dechlorination activity.

[c] NO_3^- is given in (mM).

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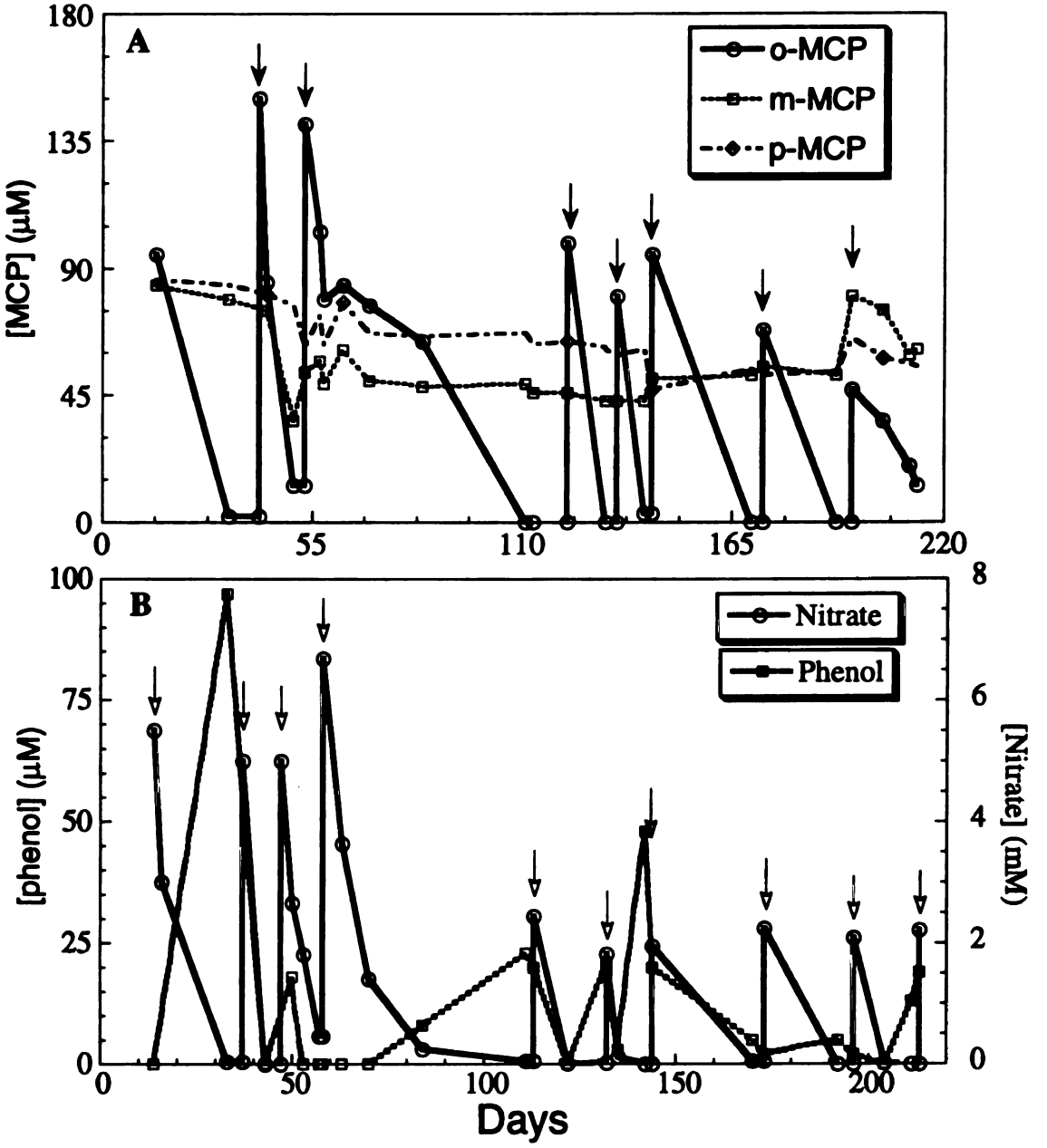

Figure 2.1. Degradation of chlorophenol in the MCP compost soil microcosm showing ortho-dechlorination activity. *o*-MCP dechlorination and persistence of *m*-MCP and *p*-MCP (A). Denitrification activity and transient appearance of phenol. Filled arrows indicate addition of *o*-MCP (B). Open arrows indicate the addition of fresh nitrate. Nitrite was not detected in the enrichment.

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concentrations as a degradation intermediate in this microcosm. When excess nitrate (7 mM) was amended to the microcosm on about day 55, the dechlorination rate was considerably reduced. However, when subsequent additions of only 2 mM nitrate were made to the microcosm after nitrate depletion, both dechlorination and denitrification occurred concurrently. Other microcosms exhibited similar sensitivity to nitrate, but at a lower concentration of 1 mM (data not shown). Nitrite was not detected in any of the denitrifying microcosms.

The O1C microcosms with the MCP mixture dechlorinated both *o*-MCP and *m*-MCP stoichiometrically to phenol (Table 2.1). This activity took between 140 and 200 days to develop and only occurred when nitrate was completely depleted. Upon refeeding, both *o*-MCP and *m*-MCP were dechlorinated simultaneously (Figure 2.2). The addition of nitrate after 380 days slowed the dechlorination rate, but nitrate was rapidly consumed and N_2 was detected in the Ar-purged headspace. This indicated that an active denitrifying population was present despite the absence of nitrate for nearly 300 days in the microcosm. The persistence of *p*-MCP suggests that abiotic loss of CPs was not a factor in this culture.

Two microcosms amended with mixtures of DCPs also showed dechlorination activity and similar sensitivity to nitrate concentrations, however there was little selectivity for which DCPs were dehalogenated. Several aromatic dechlorination products were observed in the microcosms, including all of the MCPs (Table 2.1). Phenol, *o*-MCP, *m*-MCP and benzoate appeared transiently after several subsequent additions of the DCP mixture. *Para*-chlorophenol continued to accumulate in the compost DCP microcosm, but to only 28% of the theoretical concentration that would occur if no *para*-dechlorination of the isomers had occurred.

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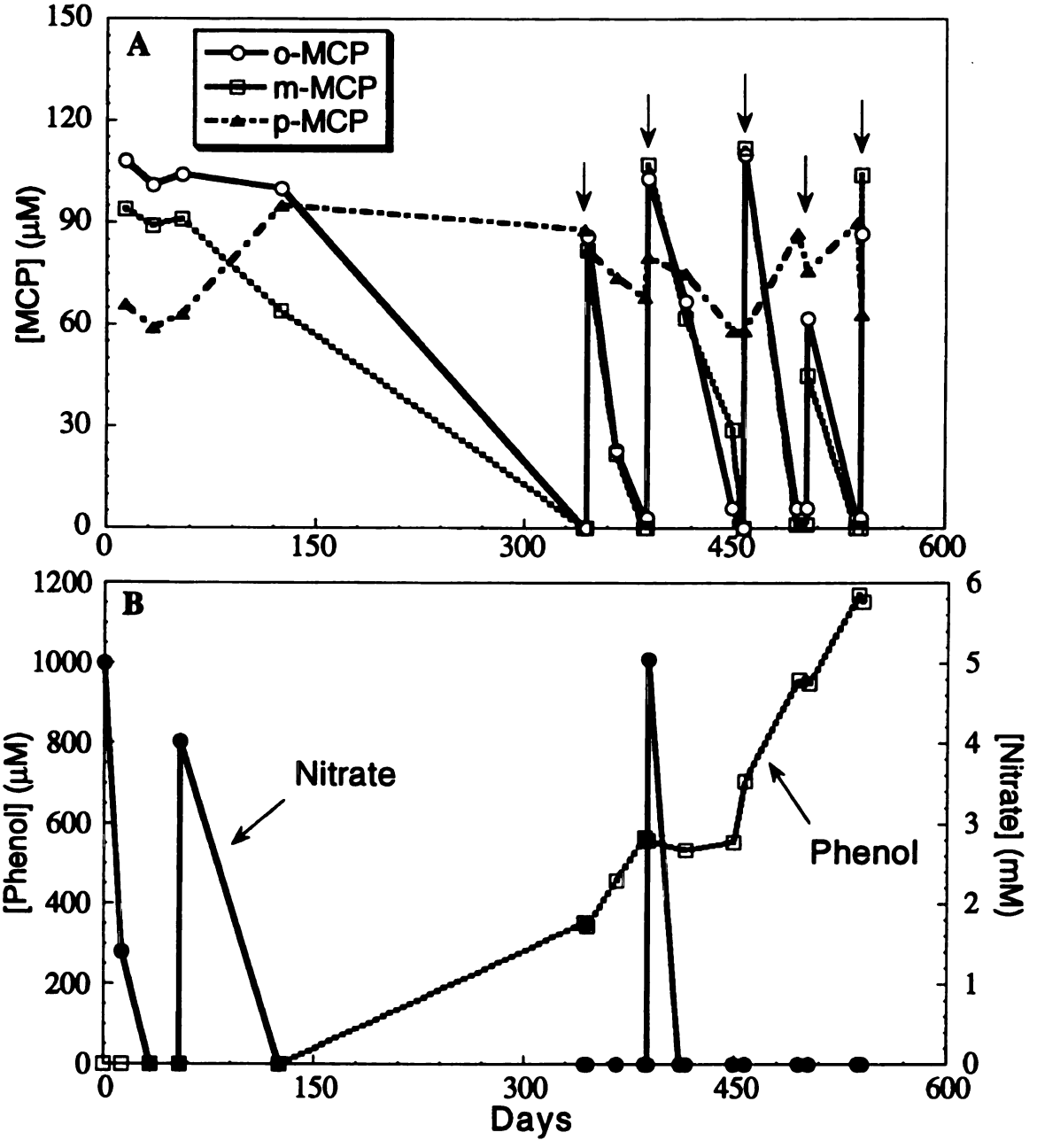

Figure 2.2. Dechlorination of *o*-MCP and *m*-MCP in the O1C MCP microcosm. (A) Concentrations of MCPs over 550 days of incubation. Closed arrows indicating the addition of *o*-MCP and *m*-MCP. (B) Indicates the concentrations of phenol and nitrate as they accumulate and dissappear respectively.

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Independence of dechlorination from nitrate reduction. Since several of the above experiments suggested dechlorination occurred prior to mineralization and that nitrate above 5 mM was inhibitory to dechlorination, we tested directly the effect of nitrate on dechlorination in enrichments derived from the above microcosms. Degradation of MCPs to phenol occurred in enrichments when acetate was supplied as an electron donor, however no degradation occurred when nitrate was added. Figure 2.3A illustrates transfers of the 1 mM nitrate microcosm to a fresh medium with and without nitrate. In the presence of nitrate and no additional electron donors no degradation was seen. With acetate as an electron donor, this *o*-MCP enrichment stoichiometrically dechlorinated *o*-MCP to phenol. Phenol concentrations reached greater than 2 mM as long as acetate was present. Similar results were obtained in enrichments inoculated from other microcosms exhibiting *o*-MCP dechlorinating activity. When both acetate and nitrate were present dechlorination activity also continued in these *o*-MCP enrichments (data not shown). In contrast, nitrate was inhibitory to all other dechlorination activities, even in enrichments also containing acetate (Table 2.2). Transfers of the DCP microcosms with *meta*-dechlorination activity to media containing *m*-MCP with and without nitrate also showed the same response. *Meta*-chlorophenol was dechlorinated to phenol with acetate present as an electron donor. The culture with nitrate and m-MCP did not have any activity (Figure 2.3B). *Meta* dechlorination was considerably slower than *ortho* dechlorination, taking almost 100 days. Similar results were obtained with enrichments containing 2,3-DCP which exhibited *ortho*-dechlorination in the presence of acetate. Subsequent *meta*-dechlorination was also observed in these enrichments after a considerable lag time. In contrast, transfers done from microcosms to a phenol plus nitrate medium showed complete phenol degradation (Figure 2.3C). These cultures exhibited extensive gas production in

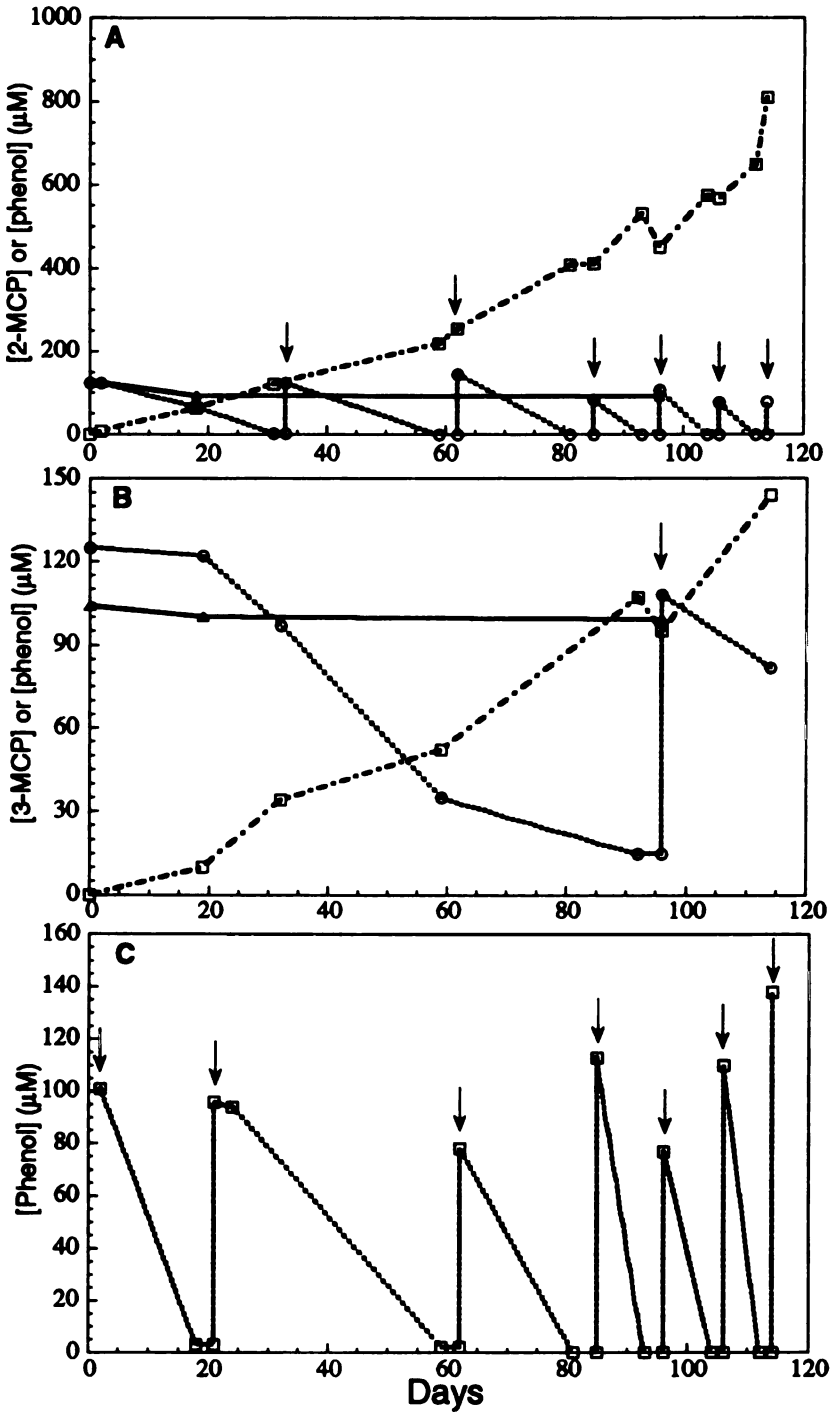

Figure 2.3. Transfers of dechlorinating and denitrifying enrichments to media with MCP plus 1 mM nitrate (▲) or 1 mM acetate (○). Phenol (□) is shown as a product of dechlorination or the substrate for degradation. (A) Dechlorination activity in *o*-MCP enrichment from Trop I microcosm. (B) *Meta* dechlorination in the DCP enrichment culture from the compost microcosm. (C) Enrichment with phenol and nitrate (1 mM) from compost microcosm Arrows denote addition of substrate.

Table 2.2 Characteristics of active enrichment cultures. At least five different dechlorination patterns are observed in the enrichments. Shown are the chlorophenolic substrate specificities of the different enrichment cultures. Also indicated is the sensitivity of dechlorination to nitrate and whether methane is detected in the enrichment cultures.

Substrate(s)[b]	Enrichments[a]				
	o-MCP culture	2,3-DCP	*o*- and *m*-MCP	*m*-MCP	3,4-DCP
o-MCP	+	-	+	-	-
m-MCP	-	+	+	+	+
p-MCP	-	-	-	-	-
2,3-DCP--> *m*-MCP	-	+	-	-	-
2,4-DCP--> *p*-MCP	+	-	+	-	-
2,5-DCP-->phenol	-	-	+	-	-
2,5-DCP--> *m*-MCP	+	-	+	-	-
2,6-DCP-->phenol	+	-	-	-	-
2,6-DCP--> *o*-MCP	+	+	-	-	-
3,4-DCP--> *m*-MCP	-	-	-	-	+
Dechlorination with					
NO_3^-[c]	+	-	-	-	-
methanogenesis[d]	-	+	-	+	+

[a] Enrichments are identified by the substrate used for dechlorination. Since *m*-MCP is a product in the 2,3-DCP and 3,4-DCP cultures the *m*-MCP dechlorinating enrichment probably is a subset microbial population of these other dechlorination enrichments.

[b] Substrates are chlorinated phenolic compounds monitored for dechlorination in cultures amended with acetate as and electron donor.

[c] 5 mM nitrate was added to media containing the primary chlorinated substrate of the enrichment and acetate. A positive result indicates that nitrate is not inhibitory to dechlorination.

[d] Positive indicates that CH_4 was detected in the headspace of enrichment cultures showing dechlorination activity.

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each culture and nitrate was completely depleted, indicating that denitrifiers were capable of degrading phenol (data not shown). Cultures with phenol alone also showed degradation activity with the appearance of methane, however this activity was considerably slower than was exhibited with nitrate.

Characterization of enrichment activity. Although dechlorination activity in all enrichments was dependent on the presence of acetate as an electron donor, there appeared to be different responses to the MCPs and DCPs added as substrates. A possible explanation was that different microbial populations were mediating reductive dechlorination in each enrichment culture. To test this, each enrichment was tested for its ability to dechlorinate the MCPs and DCPs originally used in the microcosms. In addition 2,6-DCP was used as a test substrate. Each of the five enrichments showed a different substrate specificity, indicating that five different microbial populations were responsible for dechlorination (Table 2.2). This physiological diversity between the enrichments was confirmed by testing a variety of electron donors for reductive dechlorination. Although all enrichments used acetate as an electron donor, subsequent enrichment transfers showed that this was not the best carbon and energy source for all of the dechlorinating populations. Several of the enrichments exhibited higher levels of dechlorination activity when formate, H_2, or butyrate were used as electron donors (data not shown). This would be expected among diverse microbial populations. Methanogenesis also occurred in some enrichments and it was not resolved whether this activity was correlated to the dechlorination observed in these cultures. Nitrate (1 mM) was inhibitory to dechlorination in all enrichments except the *o*-MCP enrichment (Table 2.2). Nitrate was shown to be preferentially reduced to nitrite when it was added to the 2,3-DCP enrichment and no dechlorination was observed during nitrate reduction.

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Discussion

Dechlorination of MCPs and DCPs in the microcosms occurred in the presence of nitrate, but was not coupled to denitrification. This is clearly evident since dechlorination activity in the enrichments was maintained in the absence of nitrate as long as a suitable electron donor was present. Previous studies have shown that denitrifying microcosms failed to sustain the dechlorination of chlorophenols when amended with new substrates or transferred to fresh nitrate media (Genthner et al. 1989; Häggblom et al. 1993). It was suggested in one study that nitrate at levels of 15 mM was completely depleted in the original microcosm before dechlorination occurred (Genthner et al. 1989). These authors did not report monitoring the nitrate levels in these cultures, but did report that the readdition of nitrate inhibited the dechlorination activity. This is consistent with the inhibition of dechlorination activity we observed in both the microcosms and enrichment cultures. The use of low initial nitrate concentrations along with maintaining nitrate-limiting conditions were essential to our observed concurrent and sequential denitrification and dechlorination. My results indicate that when nitrate concentrations are high (> 5 mM) in the microcosms MCP and DCP degradation rates are slowed significantly. However, as long as nitrate is removed prior to its readdition at low concentrations, the degradation of the chlorinated phenols proceeded uninhibited. Enrichment cultures are even more sensitive to nitrate, suggesting that the microcosm environment has a buffering effect on this inhibitory activity allowing dechlorination to continue. The reason other studies have failed to observe concurrent dechlorinating and denitrifying activity in chlorophenol enrichments may be because of the excessive nitrate concentrations used, generally 15 mM or greater (Genthner et al. 1989; Häggblom et al. 1993). High nitrate concentrations are contrary to what would

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naturally be present in the environment, and low levels would provide a more tolerant environment for these two processes.

Based on the appearance of phenol and chlorophenols as products of dechlorination in the microcosms, indicates that this activity is reductive (Suflita et al. 1982). This is further supported by the enrichment cultures where stoichiometric concentrations of dechlorinated phenolic products were observed in the medium if electron donors were provided. Whether individual dechlorinating populations are gaining energy through the use of the chlorophenols as physiological electron acceptors has yet to be determined in these cultures. However it is a reasonable possibility since the free energy available from the dechlorination of MCPs and DCPs is about -160 kJ/rxn (Dolfing and Harrison 1992), which is similar to that available from the reduction of nitrate to nitrite(Thauer et al. 1977). The ability for acetate to serve as an electron donor in the enrichments also suggests that halorespiration may be occurring since the oxidation of acetate is not exergonic unless it is coupled to reductive dechlorination. It has been established that *Desulfomonile tiedjei* DCB-1 does couple reductive dechlorination of 3-chlorobenzoate to energetic gain (Dolfing 1990; Mohn and Tiedje 1990). Recently Cole and co-workers isolated an *o*-MCP dechlorinating culture which also couples growth to reductive dechlorination establishing that chlorophenol respirers exist (Cole et al. 1994). The microbial populations in the current study mediate this dechlorination only when suitable electron donors are present, a feature consistent with chlororespiration. The isolation of a trichlorophenol (TCP) and DCP dechlorinating anaerobic spore former was recently reported, although its ability to grow with halorespiration was not established (Madsen and Licht 1992). Another gram-positive 2,4-DCP dechlorinating bacterium, *Desulfitobacterium dehalogenans*, was isolated from enrichments that had been heat-treated,

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implying the presence of spores (Utkin et al. 1994). This strain also was not shown to grow using chlorinated phenolic compounds as its sole physiological electron acceptor.

The dechlorination activities observed in this study are summarized in the form of a possible food web in Figure 2.4. Our evidence suggests that each of these activities may be carried out by a different population, indicating considerable diversity in chlorophenol dechlorinating organisms. It is important to recognize the different dechlorinating activities of each community before focusing on isolation, so that a broader group of isolates, some with novel activities, can be sought. Figure 2.4 suggests possible degradation pathways for the MCPs and DCPs in the enrichments studied. Even though some chlorophenols are used by more than one enrichment culture, there are substrate specificities that differentiate these cultures so that they could co-exist in an environment where a mixture of substrates exist. Those specific reductive dechlorinations mediated by different enrichment cultures would potentially cross-feed each other in the overall microbial community. Enrichment culture-specific dechlorination reactions are numbered to indicate each potentially distinct microbial population. The compost soil microcosms and subsequent enrichment cultures are able to mediate four of the five suggested dechlorination reactions as well as both denitrifying and methanogenic degradation pathways for phenol. Step one, involving *o*-MCP dechlorination to phenol, has been shown to be independent of the dechlorination of *m*-MCP (step 5) or the dechlorination of the 3,4-DCP (step 4), which involves an initial *para* dechlorination. It might be reasoned that the *ortho* dechlorinating population would dechlorinate all the DCPs, however the *o*-MCP (step 1) cultures were not able to dechlorinate 2,3-DCP (Table 2.2). As would be expected, a separate enrichment culture exhibits an alternative *ortho* dechlorination pathway which did dechlorinate 2,3-DCP (step

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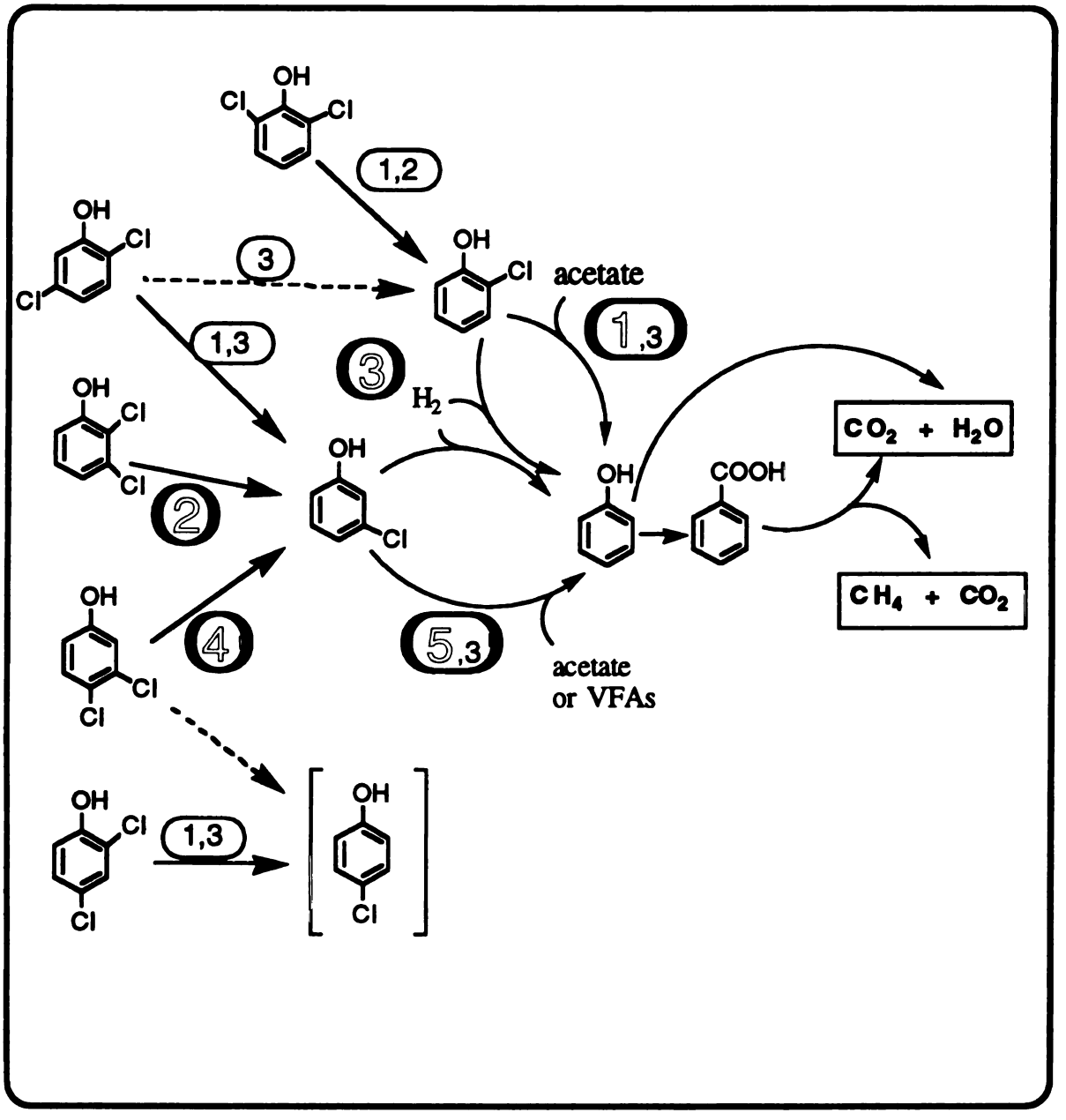

Figure 2.4. Schematic representation that summarizes the different dechlorination and degradation activities observed in the enrichments. Large numbers indicate the predominant reaction mediated by a particular enrichment and small numbers indicate additional dechlorination reactions that are observed in these cultures. (Numbers correspond to the following enrichments: reaction **1** = *o*-MCP; reaction **2** = 2,3-DCP; reaction **3** = *o*- and *m*-MCP; reaction **4** = *m*-MCP and reaction **5** = 3,4-DCP) Reactions 1, 2 and 3 represent distinct *ortho* dechlorinating pathways. Cultures mediating reaction 1 do not dechlorinate 2,3-DCP. *Meta* dechlorination is mediated by at least two different communities depicted by reactions 3 and 5. *Para* dechlorination is only observed for 3,4-DCP as shown by reaction 4. The fate of phenol is summarized in two possible pathways; denitrifying and methanogenic through benzoate.

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2). This 2,3-DCP enrichment is not able to use *o*-MCP, which indicates that the microbial populations represented in enrichments mediating step one and two could coexist when both substrates are present. Also included in Figure 2.4 is the alternative pathway for simultaneously dechlorinating *ortho*- and *meta*-substituted MCPs and 2,5-DCP (step 3). This represents a third *ortho* dechlorination reaction, perhaps mediated by hydrogen as an electron donor. This latter suggestion is derived from the transient appearance of H_2 in the headspace of enrichments in the absence of methanogenesis, suggesting a direct coupling to dechlorination. In summary the evidence suggests that there are three different *ortho* -dechlorinating populations, two different *meta*-dechlorinating populations and at least one population capable of *para*-dechlorination.

In contrast to previous studies using chlorinated phenolic compounds, mixtures of MCPs and DCPs were used, which perhaps enabled a more diverse dechlorinating community to be selected. For example although *meta* dechlorination did not occur in the compost soil microcosm with the MCP mixture, it did occur with the DCP mixture. It was even successfully transferred to enrichments with *m*-MCP as a substrate. Despite these differences the overall patterns of dechlorination in these cultures are similar to those previously observed. *Ortho* dechlorination, the most prevalent activity in our microcosms, has been observed frequently in many anaerobic cultures, although generally those have been maintained under methanogenic conditions (Boyd and Shelton 1984; Hrudey et al. 1987; Genthner et al. 1989; Kohring et al. 1989; Dietrich and Winter 1990; Hale et al. 1990; 1991; Häggblom et al. 1993). The dechlorination of *meta* substituted phenols has also been previously observed with this activity occurring at generally slower rates than *ortho* dechlorination (Boyd and Shelton 1984). The persistence of *p*-MCP is also common among other studies and our

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study supports the contention that it is the least common activity observed in methanogenic microbial enrichments (Genthner et al. 1989; Hale et al. 1990; Parker et al. 1993). However the *para*-dechlorination of 3,4-DCP to *m*-MCP in the enrichment transfer culture is the predicted activity based on the electronegativity of the chlorine in the para position which makes it the better leaving group (Cozza and Woods 1992). Previous studies, in contrast, have shown that *meta* dechlorination of 3,4-DCP is the apparent predominant biological dechlorination activity in some enrichments (Mikesell and Boyd 1985; Woods et al. 1989).

One of the interesting aspects of the results is the influence of source material on the presence of both denitrification and dechlorination. In contrast to other studies, mostly surface soils were used in our microcosms in order to favor the recovery of denitrifiers. In general, microcosms seeded with non-contaminated soils exhibit more activity than those with contaminated soil. Four of the five observed different dechlorination reactions were recovered from the compost soil microcosms. The reductive removal of chlorines from *o*-MCP was recovered in both compost and tropical soil derived enrichments. A possible selective basis for the innate dechlorination ability in these soils may be the presence of naturally occurring chlorinated phenols (Siuda and Debernardis 1973; Gribble 1992). Recently de Jong et al. (1994) established that up to 70 mg/kg of chlorinated anisyl metabolites produced by white rot fungi can be found in forest litter. They illustrated that these compounds can easily be transformed by bacteria into chlorophenols, in this case 3-chloro-anisaldehyde and 3,5-dichloroanisaldehyde to *o*-MCP and 2,6-DCP, respectively. This implies that high organic matter soils with a potentially large fungal population could be reservoirs for significant amounts of CPs.

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Although dechlorination and denitrification in the microcosms are not mediated by the same bacterial population it is possible that mineralization may be stimulated through the addition of low concentrations of nitrate. This could be advantageous in formulating remediation strategies for contaminated sites. Chlorophenolic compounds would continue to be anaerobically dechlorinated and the products will be subsequently mineralized by the denitrifiers present.

We have shown that both dechlorination and denitrification activity can be maintained in an active microcosm, but that these processes are mediated by physiologically different populations. We found no evidence for the existence of denitrifiers that dechlorinate MCPs or DCPs with nitrate reduction. Anaerobic dechlorination of MCPs and DCPs is complex, with at least five different types of reactions observed in this study. It is, however, common and under appropriate conditions, may be harnessed for use in bioremediation systems.

Acknowledgements

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Chapter III

Isolation and preliminary characterization of the aryl-halorespiring anaerobic myxobacteria strains 2CP-C and 2CP-3

Introduction

The ability to utilize haloaromatic compounds as physiological electron acceptors has only been described in a few pure cultures (Dolfing 1990; Mohn and Tiedje 1990; Cole et al. 1994; Sanford et al. 1996). Since reductive dechlorination releases considerable energy (Dolfing and Harrison 1992), the ability to halorespire in anaerobic environments is potentially beneficial to the organism catalyzing this transformation. The types of natural halogenated aromatic compounds that might be present in such an environment are many (Gribble 1992), and recently de Jong and coworkers (1994) found that white rot fungi actually generate up to 70 mg/kg of chlorinated anisyl metabolites in leaf litter. Thus, not only is a halorespiratory metabolism potentially useful, it is likely to occur in natural anaerobic ecosystems.

The reductive removal of chlorines from aromatic compounds may be important to anaerobic bioremediation strategies where considerable contamination with chlorophenolic compounds has occurred. Once chlorines are removed, the resulting aromatic compounds are generally more amenable to oxidative attack. This was observed in denitrifying microcosms in which 2-chlorophenol (2-CP) was dechlorinated by one microbial population to phenol, which was then degraded by denitrifiers (Chapter 2). Similar results have been observed with anaerobic methanogenic systems that are fed pentachlorophenol (PCP) or other poly-chlorinated phenolic compounds (Mikesell and Boyd 1986).

Many studies have shown the reductive dechlorination of PCP to di- and mono-CPs (Nicholson et al. 1992). These lesser-halogenated products of dechlorination more easily degraded under aerobic environmental conditions.

In this study, two novel anaerobic myxobacteria strains that are capable of growth using 2-CP as a physiological electron acceptor were isolated and characterized. A similar anaerobic halorespiring myxobacteria was recently described by Cole and others (1994). These strains have been named *Anaeromyxo dehalogenans*, for their novel anaerobic life-style and ability to dehalogenate chlorophenols. As a group of microorganisms, the myxobacteria have always been considered obligately aerobic microorganisms (Reichenbach and Dworkin 1992; Reichenbach 1993). However the phylogenic placement of this group, within the delta-Proteobacteria, would suggest the possibility of an ancestral anaerobic life-style for these microorganisms (Shimkets and Woese 1992). For example, sulfate-reducers and other anaerobic microorganisms are located within this group and exhibit considerable anaerobic physiological diversity. It is possible that these facultatively anaerobic myxobacteria have growth requirements that have made it difficult to isolate similar organisms in the past. The findings presented here suggest a possible life-style previously underinvestigated in the microbial world, that of the microrespirotroph. Such an organism, as observed with the *Anaeromyxo dehalogenans* strains described here, does not grow well when high concentrations of an electron acceptor are present. Novel culturing strategies may be necessary for isolating these types of microorganisms.

Materials and Methods

Isolation. Cultures were enriched from anaerobic microcosms containing different soils and mixtures of mono-chlorophenols in the presence of nitrate (Chapter 2). Enrichments were obtained with the ability to

dechlorinate 2-CP using acetate as the sole carbon and energy source. To obtain pure cultures, the agar-shake technique was used with 10 ml of 2.0 % low melting-point agarose containing 1 mM acetate and 0.2 mM 2-CP. After several weeks of incubation colonies were removed using a sterile syringe, with anoxic conditions maintained by continuous flushing of the culture tube with O_2-free N_2. The harvested colonies were transferred to broth culture containing 100 µM 2-CP and 1 mM acetate. Broth cultures that exhibited dechlorination activity were amended with more 2-CP once it was consumed. When phenol concentrations exceeded 2 mM, cultures were transferred to fresh medium. Broth cultures were used to inoculate agar plates that were degassed for at least 24 hours in an anaerobic glove box containing an N_2:H_2 gas mixture of 97:3. Plates were incubated in the anaerobic glove box and single colonies were transferred to new agar plates. After at least three transfers, colonies were transferred into chlorophenol or fumarate (5 mM) broth for further testing. Cultures were subsequently grown on plates at 30° C in an anaerobic growth vessel with an atmosphere of 10-20% CO_2 and the balance N_2.

Growth medium. Cultures were grown in 160 ml serum bottles with 100 ml of boiled degassed medium or in anaerobic culture tubes with 20 ml of medium and closed with butyl rubber stoppers. The mineral salts medium and vitamin supplemental solution were as previously described in Chapter 2.

The plate media used for culturing the 2CP strains was R2A (Difco) agar medium supplemented with 10 mM fumarate or 5 mM nitrate as electron acceptors.

Electron donors and acceptors. The ability of the 2CP strains to reductively dechlorinate 2-CP with different electron donors was determined in duplicate in 20 ml anaerobic culture tubes incubated at 30° C. The soluble volatile fatty acids (VFAs) acetate, formate, butyrate, succinate, propionate,

fumarate, lactate, pyruvate and H_2 were tested as potential electron donors. The VFAs were added to concentrations of 0.5 or 2.0 mM. H_2 was tested by adding18 μmoles (6.6 % v/v) to the headspace of the duplicate culture tubes. 2-CP (100 μM) was added as the electron acceptor. A 0.5 % inoculum was added to each tube. The disappearance of 2-CP and appearance of phenol were monitored by HPLC. 2-CP was replenished at least three times at concentrations of 100 μM to those cultures exhibiting dechlorination activity. VFA utilization was monitored by HPLC and H_2 was determined by GC. A positive test was indicated if both dechlorination and depletion of electron donor were observed.

The range of electron acceptors used by the 2CP strains was determined using the same growth conditions as with the electron donor determination. With acetate (1 mM) serving as an electron donor, 100 μM of 2,3-DCP; 2,4-DCP; 2,5-DCP; 2,6-DCP; 2-CP; 3-Cl-4-HBA; 2-fluorophenol; 2-bromophenol; 2-iodophenol; 2,3,5-TCP; 2,4,6-TCP; pentachlorophenol (PCP); or 3-chloroanisaldehyde were tested as halogenated electron acceptors. Nitrate, sulfate, sulfite, thiosulfate and fumarate were also tested at concentrations of 5 mM. Tubes were inoculated with 0.1 - 0.5 % of cultures grown on acetate and 2-CP. Halogenated substrates were monitored by HPLC and were replenished once depleted. Nitrate and fumarate concentrations were measured by HPLC. Use of sulfur compounds was determined by monitoring increases in turbidity versus control cultures with no-cells. Acetate consumption was monitored by HPLC to verify physiological activity.

Nitrate effect on dechlorination. The effect of nitrate (2 mM or 5 mM) on reductive dechlorination of 2-CP was tested in anaerobic culture tubes with acetate (1 mM) as the electron donor. Duplicate cultures of the 2CP strains were inoculated and monitored for the appearance of phenol, the

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disappearance of nitrate and appearance of nitrite. In order to determine if nitrate was reduced to N_2 or ammonia, N_2 and N_2O were monitored by GC analysis. Ammonia was determined by the indophenol blue colorimetric method.

Microscopy. Microscopic observations were done using a Leitz Orthoplan 2 microscope. Colonies on plates were illuminated with a focusing beam of light and observed under the 5 X objective. Cells were observed by suspending portions of colonies and centrifuged suspensions of broth cultures in 10 µl of phosphate buffer spread on dry agarose coated slides. Cover slips were sealed with a molten mixture of 50% paraffin and 50% vaseline. Cells were observed under oil-immersion with a 60 X phase-contrast objective lens. Photographs were taken using TMAX 100 black and white film.

Hydrogen uptake and threshold determination. Hydrogen uptake was monitored in relation to dechlorination activity to test if the hydrogenase activity was closely linked to the terminal electron-accepting capacity of the 2CP strains. Hydrogen (5.0 %) was added to the headspace of 100 ml cultures in 160 ml serum bottles. 2-CP (100 µM) was added to these cultures and the H_2 concentration was monitored by GC as dechlorination occurred. After the complete dechlorination of the 2-CP, an additional amendment of 100 µM was made. To determine the threshold concentration for hydrogen maintained by the 2CP strains while dechlorinating, triplicate cultures were monitored for H_2 depletion with excess 2-CP present. Threshold concentrations of H_2 were measured using a GC equipped with a reduction gas detector from Trace Analytical, Menlo Park, CA.

Determination of fe. As an indication of energetic efficiency and evidence of halorespiration, the fraction of electrons (**fe**) from acetate used for the reductive dechlorination of 2-CP was determined. Duplicate cultures of

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each strain were grown on acetate (1 mM) and 2-CP (125 μM). Control cultures received acetate alone. Concentrations of 2-CP were monitored daily by HPLC and amendments of new 2-CP (125 μM) were made prior to the concentration reaching zero. Acetate was determined by HPLC. The **fe** was calculated by plotting the acetate concentration consumed as H_2 equivalents versus the μmoles phenol produced. Hydrogen equivalents were used since each reductive dechlorination requires one mole H_2 per mole phenol formed. The hydrogen equivalents from acetate were calculated according the following half-reaction:

$$CH_3COO^- + 4H_2O \longrightarrow 2\,HCO_3^- + H^+ + 4\,H_2$$

Thus, for every mole of acetate consumed four moles of hydrogen equivalents are generated.

Analytical procedures. Nitrate and nitrite were analyzed by HPLC using a Whatman Partisil 10 SAX column on a Schimadzu HPLC. The eluent was 50 mM phosphate buffer (pH = 3.0) pumped at a rate of 1 ml/min. UV adsorption at 210 nm was used for detection. Samples from primary enrichments were diluted 1:100 in deionized H_2O prior to nitrate and nitrite analysis. A 40 μl sample was injected by an Alcott 738 autosampler.

Chlorophenols, dichlorophenols and aromatic products of dechlorination were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was phosphoric acid (0.1 %) buffered methanol pumped at 1.5 ml/min using a gradient from 48% to 55% methanol. A Hibar RP-18 (10 μm) column was used. Detection of eluted peaks was done at 218 nm, 230 nm and 275 nm simultaneously on a UV multiwavelength detector. Samples (1 ml) from the enrichments were taken, made basic with 10 μl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 μm filters prior to HPLC analysis.

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Volatile fatty acids were analyzed using the Schimadzu HPLC with a BioRad Aminex HPX-87H ion exclusion column heated to 60°C and using 0.005 N H_2SO_4 as the eluent. Previously filtered samples were acidified to 0.25 N H_2SO_4 by adding 100 µl of 2.5 N H_2SO_4 to 900 µl of sample. Eluent was pumped at 0.6 ml/min and detection of VFAs was at 210 nm by a UV detector.

The headspace of the incubation vessels was analyzed for N_2, H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity (TCD) detector. Headspace pressure was normalized to atmospheric by venting with a needle prior to removing 0.3 ml of gas for injection into the GC. N_2O was quantified on a Perkin Elmer 910 GC with a Porapak Q column and ^{63}Ni -ECD detector. Denitrification products were measured in an Ar headspace.

Results

Isolation of 2CP strains. Enrichment cultures from two soil types exhibited dechlorination of 2-CP. One culture was of tropical origin (Cameroon) and the other from a local compost pile. Several isolated colonies were observed in agar shake cultures from both of these enrichments. Colonies, some with a red pigment, were transferred to broth culture and showed reductive dechlorination of 2-CP with acetate as an electron donor. Three cultures designated 2CP-C (from Cameroon), 2CP-3 and 2CP-5 (from compost) were isolated. 2CP-C and 2CP-3 were selected for continued study after isolation on agar plates. Figure 3.1 shows the typical pattern of dechlorination of 2-CP and consumption of acetate exhibited by 2CP-C. The other strains exhibited similar behavior. 2CP colonies grown on R2A agar medium with fumarate as an electron acceptor developed a red pigmentation after several

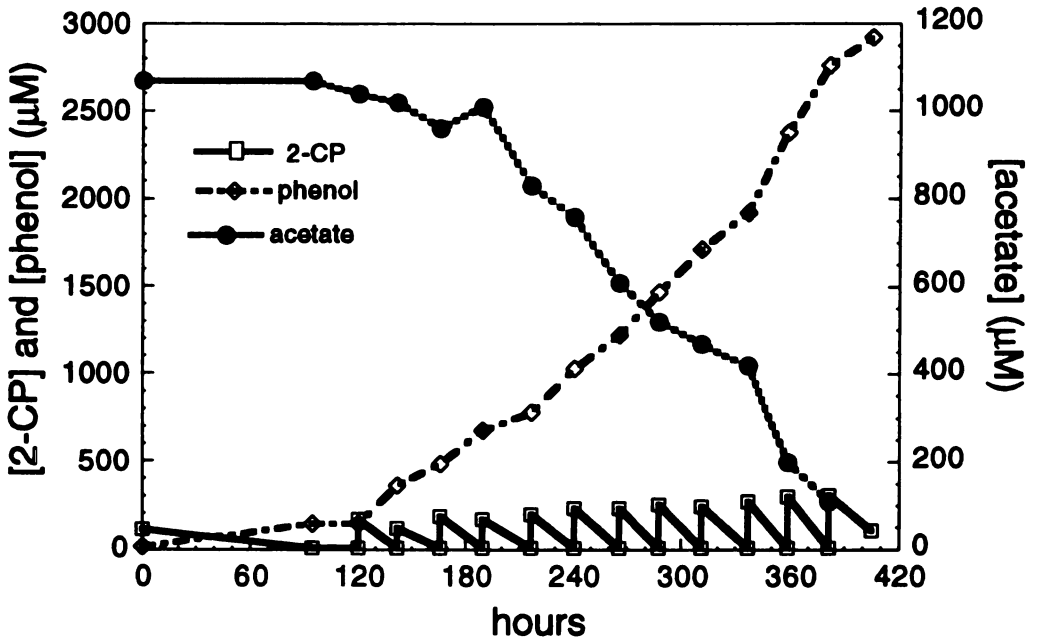

Figure 3.1. Dechlorination of 2-CP to phenol by strain 2CP-C. Reduction in the level of acetate by the same culture is also shown.

weeks of incubation. The red pigment was not extractable with acetone. Some colonies appeared to change with time, becoming more dense in pigmentation, and cells appeared to concentrate and build-up a mound at the center of the colony. These masses formed small raised colonies on the surface of the agar (Figure 3.2A). Microscopic observations of cells from this concentrated colony mass showed evidence of refractile bodies, which are indicative of spores. Vegetative cells from young cultures are shown in Figure 3.2B and some cells at this growth stage were observed to have a gliding-like motility, however no motility was seen in most of our observations. The terminal ends of cells formed hook-like structures and blebs. All 2CP strains stained Gram negative.

Electron donors and acceptors. Table 3.1 lists the general characteristics of the 2CP strains and the electron donors utilized for the reductive dechlorination of 2-CP. Acetate was the best electron donor for combined growth and dechlorination activity, although hydrogen was used in preference to acetate when both were present. The halogenated electron acceptors utilized by 2CP-C, 2CP-3 and 2CP-1 (a strain isolated by Cole (1994)) are summarized in Table 3.2. In general, all strains had the same substrate range with one exception, 2CP-C dechlorinated 2,4-DCP and 2,4,6-TCP much faster and more completely than 2CP-1. In addition to *ortho*-substituted chlorophenols 2CP-C and 2CP-1 were able to grow with 2-bromophenol as an electron acceptor. Concentrations of chlorophenols above 250 µM were inhibitory to dechlorination activity. Iodo- and fluoro-substituted halophenols were not utilized.

Nitrate, oxygen and fumarate were also used as electron acceptors by the 2CP strains (Table 3.2). Plates that were incubated with 5 % oxygen and 10 % CO_2 added to the gas mixture exhibited colony formation within one to two weeks. Aerobically incubated plates exhibited poor growth or colonies did not

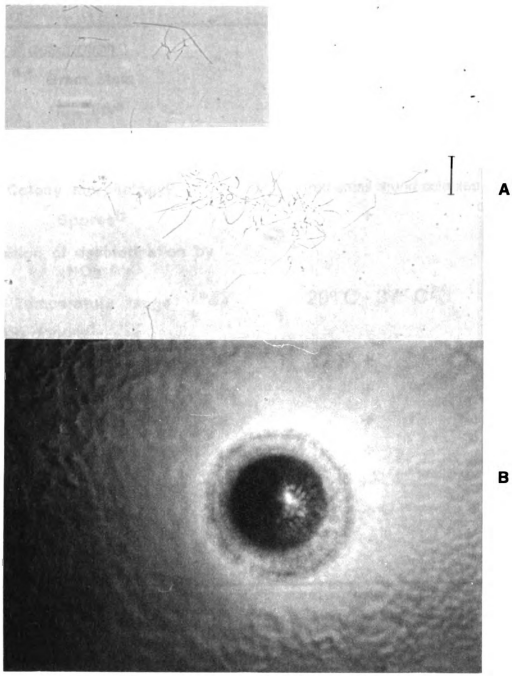

Figure 3.2. (A) Vegetative cells of strain 2CP-C by phase contrast microscopy. Bar is 13 μm long. (B) Colony formed by 2CP-C on anaerobic agar, showing the formation of a central cell-mass.

Table 3.1. Characteristics of 2CP strains. Data reflect general features observed in the 2CP strains isolated and described in this study.

General description	
Gram stain	-
Motility[a]	+
Cell morphology[b]	long slender rods with tapered ends (10 μ x 1 μ)
Colony morphology[b]	pink or red small round colonies
Spores[b]	+
Inhibition of dechlorination by NO_3^-[c]	-
Temperature range	20° C - 37° C
electron donors[d]	
Acetate	+
H_2	+
Succinate	+
Pyruvate	+
Lactate	+/-
Formate	+
Butyrate	-
Propionate	-

[a] Motility was observed in cells from a solid agar medium placed on a wet-mount agarose coated slide. Cells appeared to have gliding-type motility.

[b] Cells were grown on R2A agar plates anaerobically with fumarate as an electron acceptor. Cells were examined microscopically as described for motility.

[c] Nitrate (5 mM) did not inhibit dechlorination of 2-CP as long as acetate was present. 2-CP was used preferentially to nitrate as an electron acceptor.

[d] Electron donors were tested with 2-CP as an electron acceptor. If dechlorination was observed over two feedings of chlorophenol the substrate was scored positive.

Table 3.2. Electron acceptors used by different 2CP strains. A (+) indicates reductive dechlorination and acetate consumption occurred over at least three additions of the halogenated substrate. This indicated growth had occurred, which was confirmed by microscopic examination in those cultures marked with an (*). (+/-) indicates that activity was observed but was slow and complete degradation did not occur.

Halogenated electron acceptors	2CP-C	2CP-1	2CP-3
*2-CP	+	+	+
3-CP	-	-	-
4-CP	-	-	-
2,3-DCP	-	-	-
2,4-DCP	+	+/-	+
2,5-DCP	+	+	-
*2,6-DCP	+	+	+
3Cl-4-OH-benzoate	-	nd	nd
2,4,6-TCP	+	+/-	nd
2,3,5-TCP	-	nd	nd
PCP	-	-	nd
2-Fluorophenol	-	-	nd
*2-Bromophenol	+	+	nd
2-Iodophenol	-	-	nd
3-Cl-anisaldehyde[a]	-	nd	nd
Other electron acceptors			
*NO_3^- --> NH_4^+	+	+	+
*O_2	+	+	+
*Fumarate	+	+	+

[a] 3-Cl-anisaldehyde is a natural product produced by white-rot fungi.
nd. not determined.

grow at all. Fumarate and nitrate supported growth on plates and in broth cultures. The oxidized sulfur compounds sulfate and thiosulfate did not support growth in broth medium.

To determine if the 2CP strains were denitrifiers or were reducing nitrate to nitrite or ammonia, the fate of nitrate in cultures grown with acetate was quantified. After 21 days of incubation strain 2CP-3 converted most of the nitrate at initial concentrations of 2.5 mM and 5.0 mM into ammonia (Figure 3.3). Nitrite was also detected. Very small concentrations of N_2O and N_2 were measured, but accounted for less than 5 % of total nitrate added in the 2.5 mM culture and were insignificant in the 5 mM nitrate culture. Strains 2CP-C and 2CP-1 exhibited similar ammonia formation from nitrate, however both of these strains were not as robust in their growth on nitrate.

Dechlorination in the presence of nitrate. Since the original microcosms from which the 2CP strains were enriched contained both 2-CP and nitrate as electron acceptors, the preference exhibited by 2CP-1, 2CP-C and 2CP-3 for one or the other was determined. Figure 3.4 illustrates that dechlorination and nitrate reduction occurred in the same culture, however the preference of electron acceptors is not apparent since 2-CP was depleted completely (arrows) on three occasions during incubation. After it was depleted 2-CP was replenished to the culture. In a parallel experiment with 2-CP maintained in the cultures at all times, nitrate reduction was considerably inhibited (data not shown). Reductive dechlorination in these same cultures was not affected by the presence of nitrate at concentrations of 5 mM, indicating a preference for 2-CP as an electron acceptor.

Indicators of halorespiration. The fraction of electrons from acetate used for reducing the electron acceptor, **fe**, indicated that 64% of the electrons

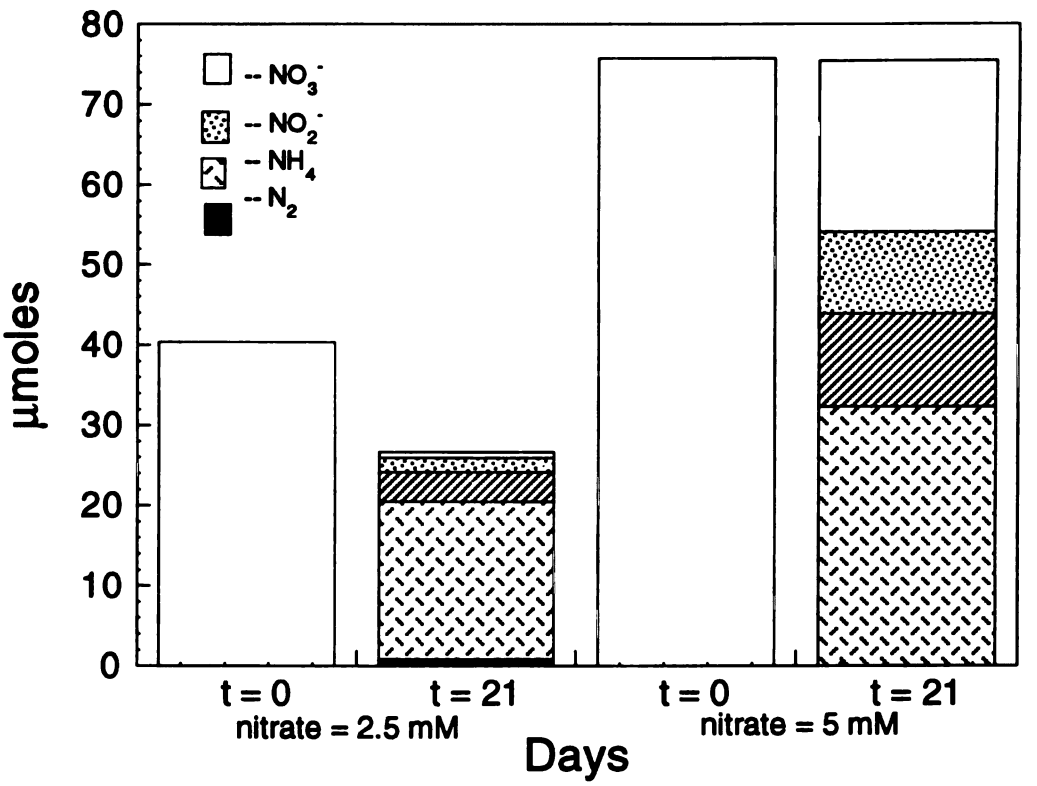

Figure 3.3. Use of nitrate by strain 2CP-3 at concentrations of 2.5 and 5 mM. Mass balance shows that most of the nitrate is converted to NH_4.

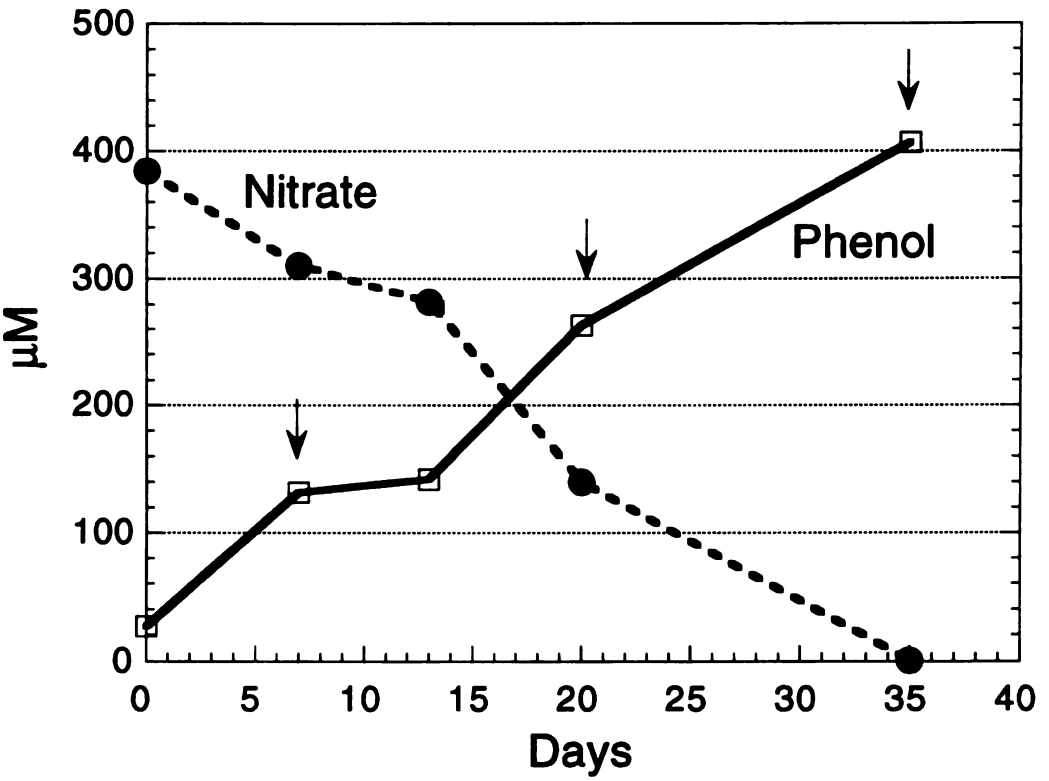

Figure 3.4. Dechlorination as indicated by phenol accumulation from 2-CP and nitrate utilization by 2CP-3. Arrows indicate when 2-CP was completely dechlorinated and when new additions of 2-CP were made.

from acetate were used for dechlorination by strain 2CP-C (Figure 3.5). Similar results were obtained with the other 2CP strains.

Hydrogen uptake and the determination of a threshold concentration for H_2 were confirmatory indicators of a halorespiratory mechanism in the 2CP strains. Figure 3.6 shows that H_2 was only assimilated in the presence of 2-CP and that the addition of this substrate immediately stimulated H_2 uptake. Threshold concentrations of hydrogen are theoretically associated with the energetic redox couple controlling their growth. The uptake of hydrogen by the 2CP strains was monitored in the presence of excess 2,6-DCP, to determine the threshold H_2 concentration (Figure 3.7). 2,6-DCP was used since the 2CP strains were able to reductively remove both chlorines and therefore an equivalent concentration had twice the electron accepting capacity of 2-CP. Within 25 hours the H_2 concentration was approximately 1 ppm and still decreasing. Final threshold concentrations after 48 hours of incubation, of less than 0.5 ppm were measured for the 2CP strains (data not shown).

Discussion

Strains 2CP-C and 2CP-3 along with the previously isolated 2CP-1 represent a new group of facultatively anaerobic myxobacteria (Cole et al. 1994). Phylogenetically they are most similar to the *Myxococcaceae* , however their 16S rRNA secondary structure shares features with *Nanocystis* and the Chondomyces group (Cole, Sanford and Tiedje, manuscript in preparation). This indicates the possible early evolutionary branching of the 2CP strains from the well-characterized aerobic myxobacteria. Several physiological features of the 2CP strains are shared with other myxobacteria. Bright red pigments were produced by all 2CP isolates, which are common among the myxobacteria (Reichenbach and Dworkin 1992). Cell shape, bleb formation and the ability to

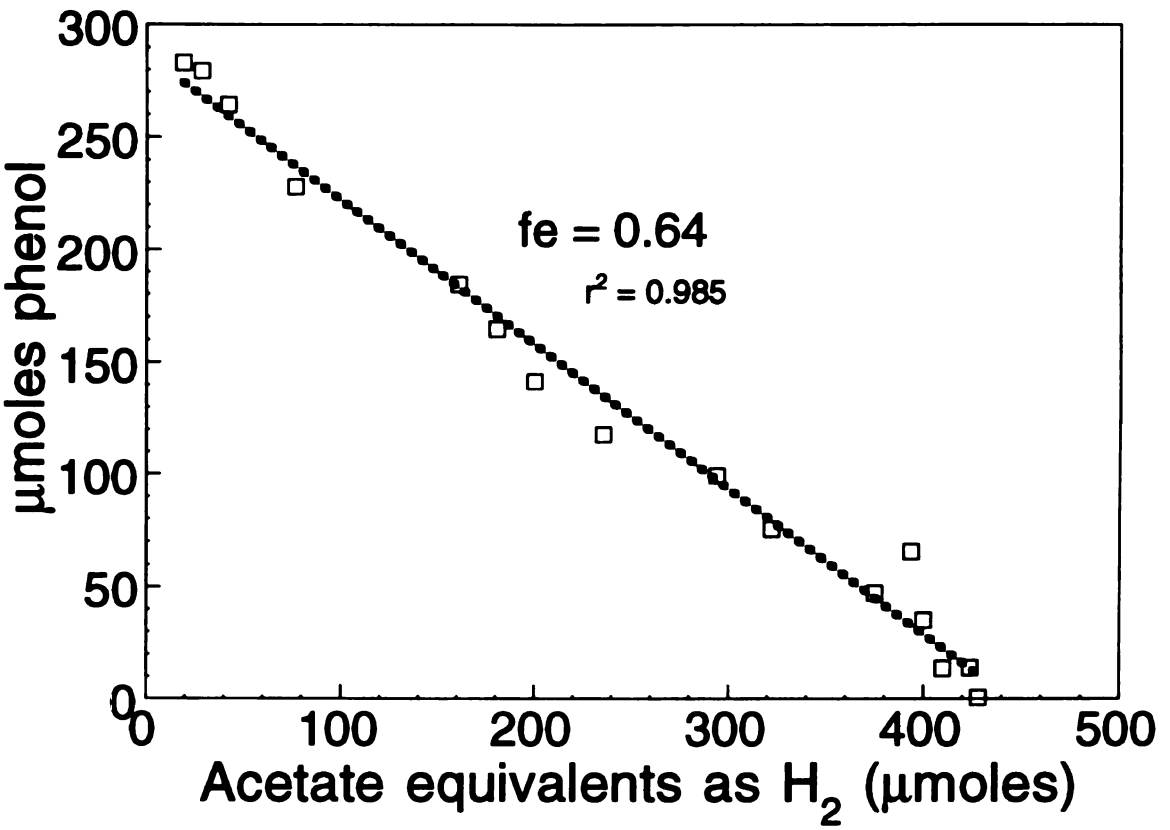

Figure 3.5. Fraction of electron (**fe**) from acetate used for reductive dechlorination of 2-CP by strain 2CP-C. The **fe** is derived from the slope of the regression line generated by plotting the acetate consumed as H_2 equivalents versus the µmoles 2-CP dechlorinated.

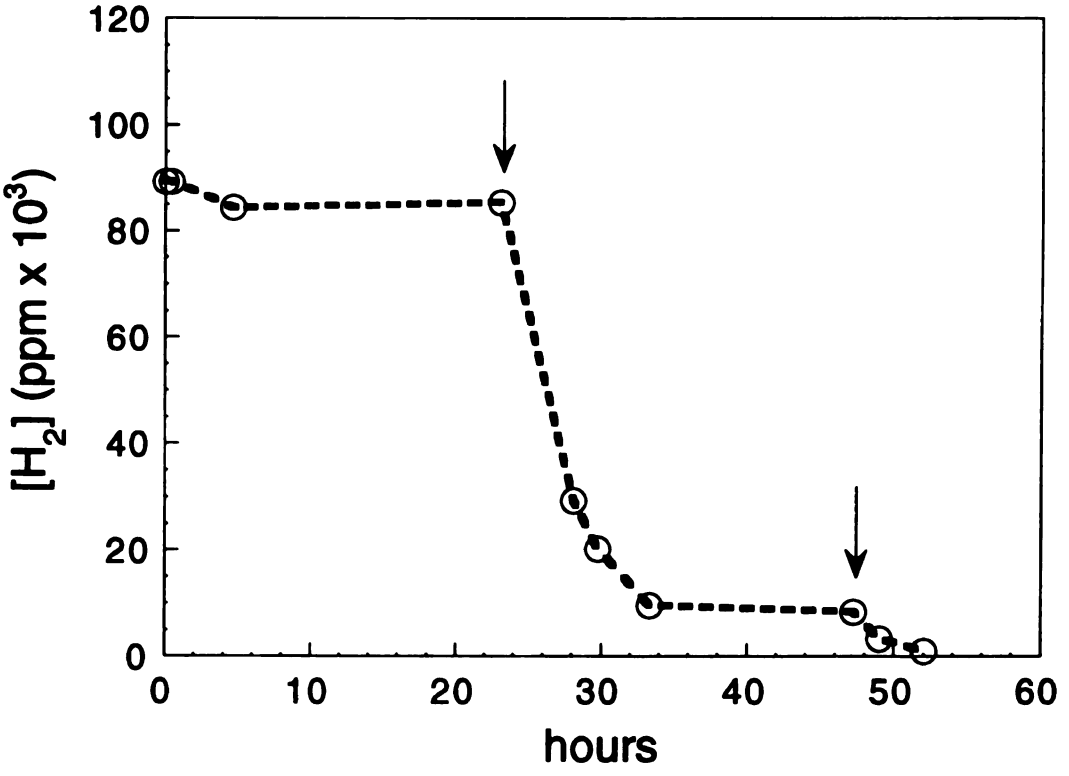

Figure 3.6. Consumption of H_2 by 2CP-C under electron acceptor limited conditions. Arrows indicate when 2-CP was added to the medium.

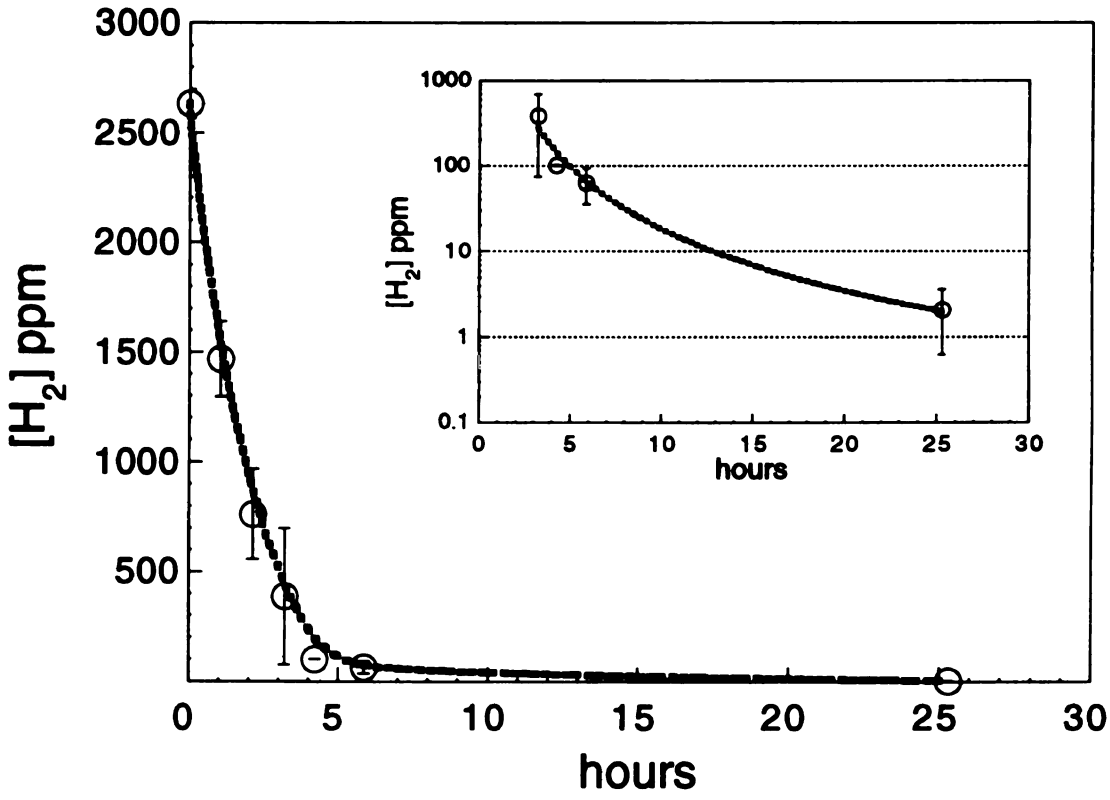

Figure 3.7. Uptake of H_2 by 2CP strains in the presence of excess 2-CP. Inset shows the threshold concentration of H_2 for this culture.

form spore-like structures are also commonly observed features (Reichenbach 1993). The colony morphology exhibited by 2CP-C, where cells appear to collect together in a raised mound is very similar to the morphology shown by *Myxococcus xanthus* fruiting-body deficient mutants (Zusman 1984). The loss of the ability to form fruiting-bodies has been reported to be common in myxobacteria, particularly if they have been maintained in broth cultures (Reichenbach 1993). As all of the 2CP strains were enriched in liquid medium, it would be reasonable to expect that well-formed fruiting bodies would not form. The gliding-like motility of the 2CP-C cells observed microscopically is also consistent with the behavior of myxobacteria (Burchard 1984). Possible reasons that this motility was not always observed is the lack of a suitable surface and inappropriate environmental conditions such as pH, O_2 tension or cell-density.

The ability of the 2CP strains to grow anaerobically on a diverse group of electron acceptors implies that the description of the myxobacteria as having strictly aerobic life-styles needs to be reevaluated. It is not necessarily unexpected to find anaerobic myxobacteria, since they are in the delta-subdivision of the Protiobacteria. This group is known for sulfate reducers, ferric reducers and other halorespirers which contribute to the group's considerable anaerobic physiological diversity.

Perhaps the most unique feature of the 2CP strain physiology is the preferential utilization of 2-CP as an electron acceptor over nitrate. Energetically nitrate reduction to nitrite has a $\Delta G^{\circ\prime}$ of -81.6 kJ mole electron^{-1} transferred (Thauer et al. 1977), while 2-CP reduction to phenol has a $\Delta G^{\circ\prime}$ of -78.5 kJ mole electron^{-1} (Dolfing and Harrison 1992). This suggests that nitrate reduction should occur in preference to reductive dechlorination. However, the $\Delta G^{\circ\prime}$ of nitrate reduction to ammonia is only -75.0 kJ mole electron^{-1}, which is

less than the free-energy from the dechlorination of 2-CP. If the electron acceptor of choice was only mediated by the thermodynamics of the reaction it might be reasonable to expect the initial reduction of nitrate to nitrite, followed by reductive dechlorination and finally nitrite reduction to ammonia. The reason that the 2CP strains do not use nitrate before 2-CP may be that nitrite accumulation would be inhibitory to their growth and therefore they must reduce the nitrite further to ammonia. This apparent selective advantage of using the energy available from reductive dechlorination is indirectly suggestive of a halorespiratory metabolism for the 2CP strains. In order to gain an equal or greater benefit energetically from reductive dechlorination over the respiratory reduction of nitrate to ammonia, an electron transport chain most likely must be involved.

The ability to use acetate as the sole carbon and energy source is generally considered to be limited to the domain of respiring microbes, with the exception of acetoclastic methanogens. Acetate utilization therefore provides evidence of halorespiration in the 2CP strains. A respiratory mechanism is confirmed when hydrogen provided as an electron donor is consumed. H_2 uptake only occurs when 2-CP was present (Figure 3.6). As H_2 could serve as the electron donor for growth, the plausible explanation was that hydrogen oxidation is coupled to 2-CP reduction via an electron transport chain. Other evidence of halorespiration by the 2CP strains is apparent in the determination of the **fe** and the measurement of hydrogen uptake correlated to the presence of 2-CP. The **fe** accounts for the portion of electrons from the electron donor used for the reduction of the electron acceptor (Criddle et al. 1991). The remaining fraction of electrons is designated the **fs**; those that are incorporated into biomass synthesis. Since **fe + fs = 1**, the **fs** is calculated by subtracting the **fe** from 1. In the case of the 2CP strains grown on acetate, this gave an **fs**

of 0.36, which is less than what is expected for denitrifiers, but greater than expected for sulfate reducers (McCarty 1975; Criddle et al. 1991).

The hydrogen threshold concentration of less than 0.5 ppm also suggests that halorespiratory-mediated growth was occurring in the 2CP strains. The hydrogen threshold concentration reflects the minimum amount of energy that an organism requires to grow (Cord-Ruwisch et al. 1988; Lovley and Goodwin 1988). Hydrogen threshold concentrations are therefore correlated with the energy available from the redox couples mediating their growth. In the case of the 2CP strains, this is for H_2 oxidation and 2-CP reduction to phenol. The threshold concentration of H_2 measured is comparable to that observed for fumarate reduction and slightly greater than found for nitrate respiration (Cord-Ruwisch et al. 1988). However, it is much less than observed for sulfidogens, methanogens and acetogens. DeWeerd , Concannon and Suflita (1991) measured a comparable threshold concentration of 0.7 ppm for hydrogen with the halorespirer *Desulfomonile tiedjei* strain DCB-1 when grown on H_2 and 3-chlorobenzoate. This indicates that hydrogen threshold concentrations may be reliable indicators of halorespiratory metabolism in pure and mixed cultures where a halogenated substrate is the only electron acceptor provided other than CO_2.

One interesting feature of the microcosms from which the 2CP strains were enriched and isolated was the apparent sensitivity to nitrate concentrations greater than 5 mM (Chapter 2). 2CP-C came from a tropical soil microcosm which had no dechlorination activity above 1 mM nitrate. In growth assays, nitrate concentrations were always 5 mM or less with the 2CP strains. The use of low concentrations in enrichments and in culturing is in contrast to previous strategies for isolating such organisms, but more reasonably models in situ environmental conditions. With the exception of fumarate, the 2CP strains

only grew well when low concentrations (< 2.5 mM) of nitrate, O_2 or 2-CP were provided. It is possible that these anaerobic myxobacteria represent a previously uncharacterized life-style, that of the microrespirotroph. Such a microbial population would be sensitive to high concentrations of electron acceptors much like an oligotroph is sensitive to high concentrations of carbon substrate. The microrespirotroph life-style would also explain why microbes like the 2CP strains have not been recovered before, since enrichment and isolation methods used in the past usually provided excess electron acceptor, which by definition would inhibit the growth of such organisms. The fortuitous use of low chlorophenol concentrations, mainly for the purpose of toxicity reduction, may have led to the discovery of this life-style in the 2CP microorganisms. This may be of benefit for the development of new culturing strategies for uncovering some of the untapped microbial diversity that exists in nature.

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Chapter IV

Characterization of *Desulfitobacterium chlororespirans* sp. nov. strain Co23, which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate

The work described here was completed by Robert A. Sanford with the exception of the 16S rRNA phylogenetic analysis completed by James R. Cole and the deterination of the temperature stability of dechlorination activity done by Frank E. Löffler.

Abstract

Strain Co23, an anaerobic spore-forming microorganism, was enriched and isolated from a compost soil based on its ability to grow using 2,3-dichlorophenol (DCP) as its electron acceptor. *Ortho* chlorines were removed from poly-substituted phenols, but not from monohalophenols. Growth by chlororespiration was indicated by a growth yield of 3.24 g cells per mole of H_2 equivalents from lactate oxidation to acetate in the presence of 3-chloro-4-hydroxybenzoate (3Cl-4-HBA), but no growth in the absence of the halogenated electron acceptor. Other indicators of halorespiration were the fraction of electrons from the electron donor used for dechlorination (0.67) and the H_2 threshold concentration of <1.0 ppm. Additional electron donors utilized for reductive dehalogenation were pyruvate, formate, butyrate, crotonate and H_2. Pyruvate supported homoacetogenic growth in the absence of an electron acceptor. Strain Co23 also used sulfite, thiosulfate and sulfur as electron acceptors for growth, but not sulfate, nitrate or fumarate. The temperature optimum for growth was 37°C, however the rates of dechlorination were optimum at 45°C and activity persisted to temperatures as high as 55°C. The 16S rRNA sequence was determined and strain Co23 was found to be related to *Desulfitobacterium dehalogenans*, with a sequence similarity of 97.2%. The phylogenetic and physiological properties exhibited by strain Co23 place it as a new species designated *Desulfitobacterium chlororespirans*.

Introduction

Chlorophenolic compounds have been of environmental concern due to their extensive use in several industries and their toxicity. This has led to considerable efforts to study the degradation of these compounds under aerobic and strictly anaerobic conditions (Häggblom 1992; Mohn and Tiedje 1992). Pentachlorophenol (PCP), a widely used wood preservative, is degraded under methanogenic conditions via a series of reductive dechlorinations (Mikesell and Boyd 1986; Mikesell and Boyd 1988; Nicholson et al. 1992; Parker et al. 1993; Juteau et al. 1995) and under aerobic conditions by several pure bacterial cultures via hydroquinone intermediates (Stanlake and Finn 1982; Middeldorp et al. 1990). Other polychlorinated phenols and monochlorophenols have been observed to degrade in a similar fashion under anaerobic and aerobic conditions (Boyd et al. 1983; Gibson and Suflita 1986; Hrudey et al. 1987; Genthner et al. 1989; Dietrich and Winter 1990; Hale et al. 1990; Zhang and Wiegel 1990; Madsen and Aamand 1992).

Anaerobic dechlorination has been widely observed in nature, although few pure cultures capable of coupling growth to this process via halorespiration have been isolated (Dolfing 1990; Mohn and Tiedje 1990; Holliger et al. 1993; Cole et al. 1994). Halorespiration refers to the ability of a microorganism to utilize the considerable energy released during reductive dehalogenation as the terminal process in a respiratory electron transport chain. Free-energies ($\Delta G^{\circ\prime}$) available from reductive dechlorination have been reported in the range of -140 to -160 kJ/reaction for chlorinated aromatic compounds (Dolfing and Harrison 1992) and -130 to -170 kJ/reaction for chlorinated aliphatic compounds (Dolfing and Janssen 1994). *Desulfomonile tiedjei* strain DCB-1 uses 3-chlorobenzoate (3-CBA) as a terminal electron acceptor and was the

first organism isolated capable of growing in this manner (Shelton and Tiedje 1984). The anaerobic myxobacteria strains, 2CP-1, 2CP-2, 2CP-C and 2CP-3, all have been shown to grow at the expense of acetate oxidation and 2-chlorophenol (2-CP) dechlorination (Cole et al. ; Cole et al. 1994). More recently *Desulfitobacterium dehalogenans* , a gram-positive microorganism, was described with the ability to remove *ortho*-substituted chlorines from polychlorinated phenols (Utkin et al. 1994). Direct evidence, however, for growth associated with halorespiration was not presented for this strain, although cell yields increased in the presence of a chlorinated substrate. Tetrachloroethene (PCE) has also been shown to serve as an electron acceptor for growth of two new genera, *Dehalobacter restrictus* and *Dehalospirillum multivorans* (Holliger et al. 1993; Schlolz-Muramatsu et al. 1995).

Previously we described microcosms and enrichment cultures with the ability to dechlorinate 2,3-DCP to 3-CP and subsequently to phenol and methane (Sanford and Tiedje 1996). Here we describe the isolation of strain Co23, a spore forming relative of *Desulfitobacterium dehalogenans* , with the ability to grow using lactate as an electron donor and several haloaromatic compounds as electron acceptors.

Materials and Methods

Growth conditions. Cultures were grown in 160 ml serum bottles with 100 ml of boiled degassed medium or in 30 ml anaerobic culture tubes with 20 ml of medium and closed with butyl rubber stoppers. The mineral salts medium (Stevens et al. 1992) and vitamin solution compostion (Wolin et al. 1963) were as described previously (Chapter 2) Cultures were incubated at 30 ° C unless indicated otherwise.

The solid medium used for culturing strain Co23 was identical to the basal medium with the following additions: 15 g/l Bactoagar, 10 mM pyruvate, 1.0 g/l yeast extract and 10 mM 3Cl-4-HBA. R2A agar medium (Difco) was also used to grow Co23. Plates were incubated in an anaerobic glove-box under a 97% N_2: 3 % H_2 atmosphere.

Liquid cultures of strain Co23 were grown on the mineral medium amended with 20 mM lactate and 1 mM 3Cl-4-HBA. After dechlorination had occurred two 1.0 % transfers were made to 20 ml of media, one with lactate and 3Cl-4-HBA and one containing lactate only. Additional amendments of 3Cl-4-HBA (2 mM each time) were made after its dechlorination to 4-hydroxybenzoate (4-HBA) until the lactate had been depleted. In order to ensure continuous growth, 3Cl-4-HBA was never allowed to be completely removed. Growth was monitored by measuring absorbance in the anaerobic culture tubes at 600 nm. The cell yield was determined by dry-weight measurements from 100 ml of culture after cells had consumed 20 mM lactate. Cells were concentrated by centrifugation in corex glass tubes to a volume of 5 ml, which was subsequently filtered through a pre-weighed 0.2 μm filter. The filters were dried for 2 h at 104° C, allowed to cool in a desiccator and weighed until a constant mass was recorded.

Determination of electron donors and acceptors. The ability for strain Co23 to reductively dechlorinate 2,3-DCP with different electron donors was determined in duplicate 20 ml culture tubes incubated at 30° C. Acetate, formate, butyrate, succinate, propionate, fumarate, lactate, pyruvate, crotonate and H_2 were tested as potential electron donors. The volatile fatty acids (VFAs) were added to concentrations of 0.5 or 2.0 mM. H_2 (18 μmoles or 6.6 % v/v) was added to the headspace of the duplicate culture tubes. 2,3-DCP (100 μM)

or 1 mM 3Cl-4-HBA were added as electron acceptors. A 0.5 % inoculum was added to each tube from a butyrate, formate and 2,3-DCP grown culture. The disappearance of 2,3-DCP and appearance of 3-CP were monitored by HPLC. 2,3-DCP was replenished three times at concentrations of 100 μM to those cultures exhibiting dechlorination activity. 4-HBA appearance was monitored in cultures receiving 3Cl-4-HBA as an electron acceptor. VFA utilization was monitored by HPLC and H_2 use was determined by GC. Dechlorination and depletion of electron donor constituted a positive test for growth.

The range of electron acceptors used by strain Co23 was determined using the same growth conditions as for the electron donor determination, but with lactate (5 mM) or butyrate (5 mM) serving as the electron donors. The halogenated electron acceptors tested were 100 μM of 2,3-DCP; 2,4-DCP; 2,5-DCP; 2,6-DCP; 2-CP; 3-CP; 4-CP; 3Cl-4-HBA; 3-chlorobenzoate (CBA); o-fluorophenol; o-bromophenol; o-iodophenol; 2,3,5-TCP; 2,4,6-TCP; 2,4,6-tribromophenol; pentachlorophenol (PCP); 3-chloroanisaldehyde; 3-chloro-L-tyrosine; and 3-Cl-4-hydroxyphenylacetate. Nitrate, sulfate, sulfite, thiosulfate and fumarate were also tested at a concentration of 5 mM. Sulfur was tested as an electron acceptor by adding 0.1 ml of a powdered S° slurry to one set of tubes. Tubes were inoculated with a 0.1 to 0.5 % inoculum from cultures grown on butyrate and 2,3-DCP or lactate and 3Cl-4-HBA. The concentrations of halogenated substrates were monitored by HPLC and were replenished once depleted. Nitrate and fumarate concentrations were measured by HPLC. Use of sulfur compounds was determined by measuring growth as indicated by absorption at 600 nm as compared to a lactate-only control culture. Lactate and butyrate consumption were monitored by HPLC to verify physiological activity.

Pasteurization and microscopy. To test for the presence of heat-resistant spores, fresh anaerobic medium containing lactate and 3Cl-4-HBA was heated to a temperature of 80° C. A 1.0% inoculum from a one week old Co23 culture was added to duplicate bottles, which were immediately placed back in an 80° C water bath for 10 min. The cultures were removed from the water bath and placed in the 30° C incubator and monitored for dechlorination activity and consumption of lactate.

Microscopic observations were done as previously described in Chapter 3.

Temperature effect on growth and dechlorination. Cultures of Co23 were inoculated into anaerobic culture tubes at temperatures ranging from 15° C to 75° C with 20 mM pyruvate and 1 mM 3Cl-4-HBA. Growth was monitored by measuring absorbance in the anaerobic culture tubes at 600 nm. To determine the temperature stability of the dechlorination activity in the absence of growth, Co23 was grown at 37° C on 20 mM pyruvate and 1 mM 3Cl-4-HBA until stationary phase had been reached, about 48 h. The cell suspension was aliquoted (10 ml) into separate culture tubes which were amended with 4 mM 3Cl-4-HBA and 10 mM pyruvate and incubated at temperatures ranging from 20° to 65° C. The rate of dechlorination in these cultures was monitored over the next 3 days by HPLC analysis.

16S rRNA gene isolation, sequencing and analysis. Cells of Co23 were grown in 100 ml of lactate and 3Cl-4-HBA medium. Total DNA was isolated from Co23 by a method shown to work on diverse bacteria (Visuvanathan et al. 1989). The primers used to amplify near full length 16S rRNA gene sequences (5'AGAGTTTGATCCTGGCTCAG3' and 5'AAGGAGGTGATCCAGCC3') were modified from the primers FD1 and RD1 of

Weisberg et al. (1991) by removing the linker sequences. The PCR reaction consisted of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, 1x *Taq* polymerase buffer, 0.75 U *Taq* polymerase (Promega, Madison, WI), and 0.1 µg DNA in a volume of 30 µl. Amplification was carried out using a GeneAmp PCR system 9600 thermocycler (Perkin Elmer, Norwak, CT) with a program consisting of an initial denaturation at 92° C for 2 min 10 s, 30 cycles of 94° C for 15 s, 55° C for 30 s, and 72° C for 2 min 10 s, followed by a final elongation cycle at 72° C for 6 min 10 s.

The resulting PCR product was purified by gel electrophoresis through a 1% agarose gel and recovered using Gene Clean purification resin according to the manufacturer's suggestions (Bio 101, La Jolla, CA). The purified PCR product was cloned in the vector pCRII, using a TA cloning kit (Invitrogen, San Diego, CA). Plasmid DNA containing the 16S rRNA gene insert was isolated from one clone using the Qiagen plasmid mini kit (Qiagen, Chatsworth, CA).

The DNA sequence of the insert was determined by automated fluorescent dye terminator sequencing using an ABI Catalyst 800 laboratory robot and ABI 373A Sequencer (Applied Biosystems, Foster City, CA). Primers used corresponded to conserved regions of the 16S sequence (Woese 1987). Approximately 95% of the insert sequence was determined in both directions.

Initial phylogenetic placement was obtained using the Ribosomal Database Project (RDP) (Larsen et al. 1993) electronic mail server program SIMILARITY_RANK. Related sequences and a preliminary alignment were obtained using the RDP programs SUBALIGNMENT and ALIGN_SEQUENCE. The alignment was completed using the sequence editor GDE obtained from the RDP. A maximum-likelihood phylogenetic tree was created using the fastDNAml (Olsen et al. 1994), using a weighting mask to include only

unambiguously aligned positions with all other program options at their default values. This analysis was repeated on 100 bootstrap samples to obtain confidence estimates on the branching order (Felsenstein 1985). The program consense from PHYLIP (Felsenstein 1989) was used to determine the number of times that each group shown in the final tree was monophyletic in the bootstrap analysis. The final tree was then produced using the program TreeTool from the RDP.

The 16S rRNA sequence of *Clostridium* sp. DMC (EMBL accession no. X86690) was obtained from the European Bioinformatics Institute web server (www.ebi.ac.uk). The following sequences were obtained from the RDP (GenBank accession number in parenthesis): *Desulfotomaculum orientis* str. Singapore I (M34417), *Desulfitobacterium dehalogenans* str. JW/IU-DC1 (L28946), *Peptococcus niger* (X55797), *Heliobacterium chlorum* (M11212), *Desulfotomaculum nigrificans* (X62176), *Desulfotomaculum ruminis* str. DL (M34418), *Bacillus subtilis* str. 168 (K00637 M10606 X00007), and *Escherichia coli* (J01695).

Chemical analyses. Chlorophenols, dichlorophenols and aromatic dechlorination products were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was phosphoric acid (0.1 %) buffered methanol pumped at 1.5 ml/min using a gradient from 48 % to 55 % methanol. A Hibar RP-18 (10 µm) column was used. Peaks were quantified at 218 nm on a UV multiwavelength detector. Samples (1 ml) from the cultures were taken, made basic with 10 µl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 µm filters prior to HPLC analysis.

Nitrate and nitrite were analyzed by HPLC using a Whatman Partisil 10 SAX column on a Shimadzu HPLC system. The eluent was 50 mM phosphoric acid buffer (pH = 3.0) pumped at a rate of 1 ml/min. UV adsorption at 210 nm was used for detection.

Volatile fatty acids (VFAs) were analyzed using the Shimadzu HPLC with a BioRad Aminex HPX-87H ion exclusion column heated to 60°C and using 0.005 N H_2SO_4 as the eluent (Guerrant et al. 1982). Previously filtered samples were acidified to 0.25 N H_2SO_4 by adding 100 µl of 2.5 N H_2SO_4 to 900 µl of sample. Eluent was pumped at 0.6 ml/min and detection of VFAs was at 210 nm by a UV detector.

The headspace of the cultures was analyzed for H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity detector (TCD). Headspace pressure was normalized to atmospheric by venting with a needle prior to removing 0.3 ml of gas for injection into the GC. Threshold concentrations of H_2 were measured using a GC equipped with a reduction gas detector from Trace Analytical, Menlo Park, CA.

Chemicals. Chlorophenols, dichlorophenols, 3Cl-4-HBA, and 3-Cl-4-hydroxyphenylacetate were obtained from Aldrich Chemical Co. 4-HBA and 3-Cl-L-tyrosine were purchased from Sigma Chemical Co. 3-Chloro-anisaldehyde was provided by Roberta Fulthorpe, University of Toronto.

Results

Isolation of *Desulfitobacterium chlororespirans* strain Co23. Enrichment cultures inoculated from microcosms of compost soil (Chapter 2) that exhibited complete dechlorination of 2,3-DCP were fed with 2,3-DCP and acetate and accumulated 3-CP. 2,3-DCP repeatedly added to this enrichment

culture was completely dechlorinated in the *ortho* position. This activity was retained after transfer to new enrichment medium. Anaerobic agar shake cultures inoculated with the secondary enrichments yielded isolated colonies. Several colonies that were transferred to anaerobic broth medium containing butyrate and formate dechlorinated 2,3-DCP. A survey of electron donors of one of the cultures showed that acetate no longer supported dechlorination of 2,3-DCP. Since 3-CP was inhibitory to growth and dechlorination activity at concentrations above 1 mM, additional chlorinated substrates were evaluated which would not yield inhibitory products and therefore result in higher cell yields. 3Cl-4-HBA was shown to be the optimum electron acceptor for growing the culture since the product of dechlorination, 4-HBA, was not inhibitory at concentrations below 20 mM. The culture was then purified by dilution of dechlorination activity to extinction in medium containing 3Cl-4-HBA, butyrate and formate. The purity of the resulting culture, designated strain Co23 (for compost soil origin), was confirmed by streaking culture fluid onto agar medium. Only one colony type was observed on both 3Cl-4-HBA and R2A agar media. Representative isolated colonies were transferred back into broth culture and shown to dechlorinate 3Cl-4-HBA.

The isolate is a motile, gram negative-staining, curved bacillus 3 to 4 μm long by 1 μm wide (Figure 4.1). Terminal spores are present in some cells. To evaluate whether the refractal bodies (Figure 4.1) were heat resistant spores, duplicate cultures of Co23 were pasteurized. After exposure to 80° C, dechlorination and growth resumed after one week of incubation, confirming strain Co23's ability to survive the heat treatment.

Evidence for halorespiration. The ability of strain Co23 to couple reductive dechlorination to growth was determined by measuring increases in

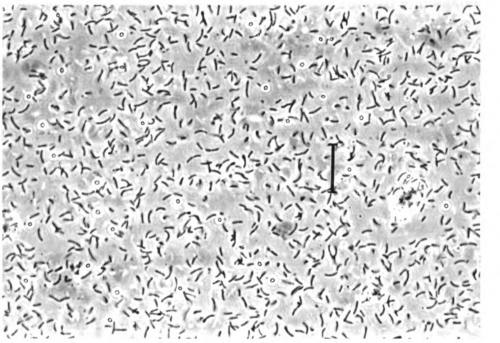

Figure 4.1. Phase-contrast photomicrograph of strain Co23. Vegetative cells and the presence of spores and terminal swelling in cells developing spores are shown. Reference bar is 13 μm.

cell density in the presence and absence of 3Cl-4-HBA. The cell density increased in lactate-fed cultures only when 3Cl-4-HBA was added (Figure 4.2). Stoichiometric generation of 4-HBA and acetate was observed from the 3Cl-4-HBA and lactate, respectively. Lactate was not oxidized in the absence of an electron acceptor. The pH was periodically adjusted with 2 N KOH to 7.2 during growth because dechlorination activity was inhibited when the pH reached 6.2.

Additional evidence of halorespiration was provided in measurements of the hydrogen threshold and the fraction of electrons from the electron donor (**fe**) being used to reduce 3Cl-4-HBA. Hydrogen was consumed within a week to a threshold level of less than 1 ppm in duplicate cultures with excess 3Cl-4-HBA present in the medium. The **fe** was determined by plotting the equivalent lactate concentration as μmoles H_2 used for oxidation to acetate versus the μmoles 4-HBA generated in the culture (Figure 4.3). The slope of the resulting regression line is equivalent to the **fe**, which was determined to be 0.67. Values of 0.69 were calculated with cultures that used butryate and formate as electron donors.

The mass balance of substrate use and cell yield when grown on lactate plus 3Cl-4-HBA is shown in Table 4.1. The cell-yield expressed as H_2 equivalents used from growth on lactate was 3.23 g cell dry weight/mole H_2 equivalent and the yield expressed on the basis of 3Cl-4-HBA dechlorinated was 6.90 g cell dry weight/ mole 3Cl-4-HBA. With butyrate and formate as electron donors the yield was 1.69 g cells/ mole H_2 equivalent.

Range of electron donors and acceptors used. Strain Co23 was capable of using a wide range of electron donors for reductive dechlorination (Table 4.2). Several substrates supported growth of strain Co23 with 3Cl-4-HBA or 2,3-DCP as electron acceptors. Growth was indicated by the measured

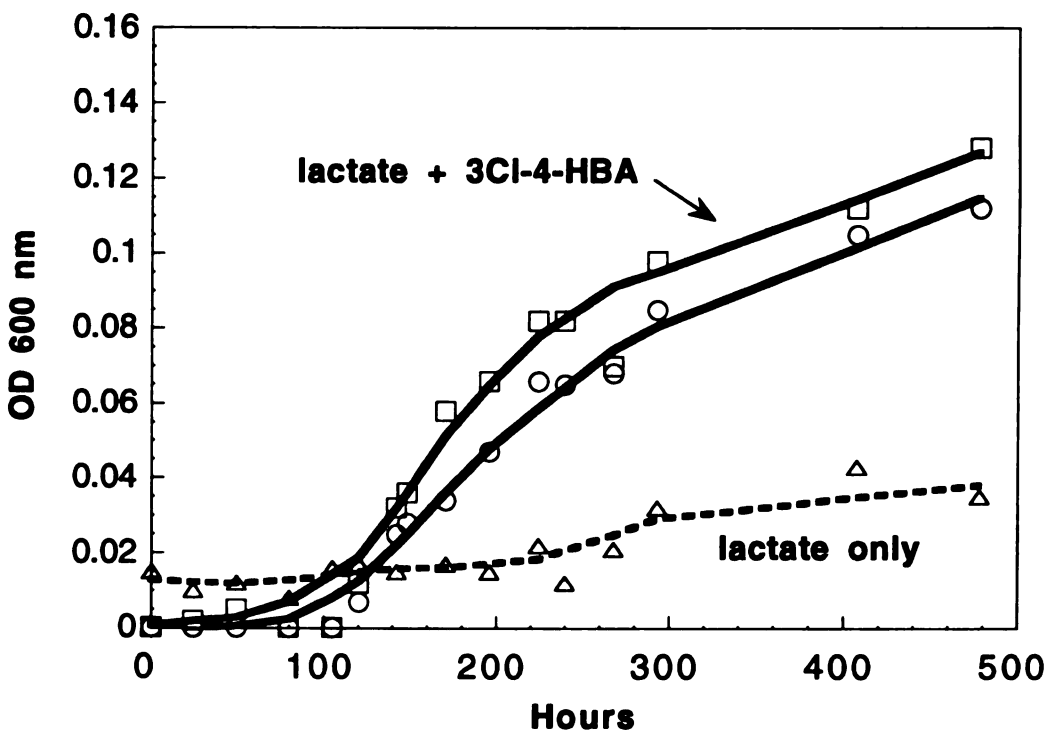

Figure 4.2. Growth of strain Co23 as indicated by an increase in turbidity (OD 600 nm). Duplicate cultures with lactate and 3-Cl-4-HBA are shown compared to a control culture containing lactate alone.

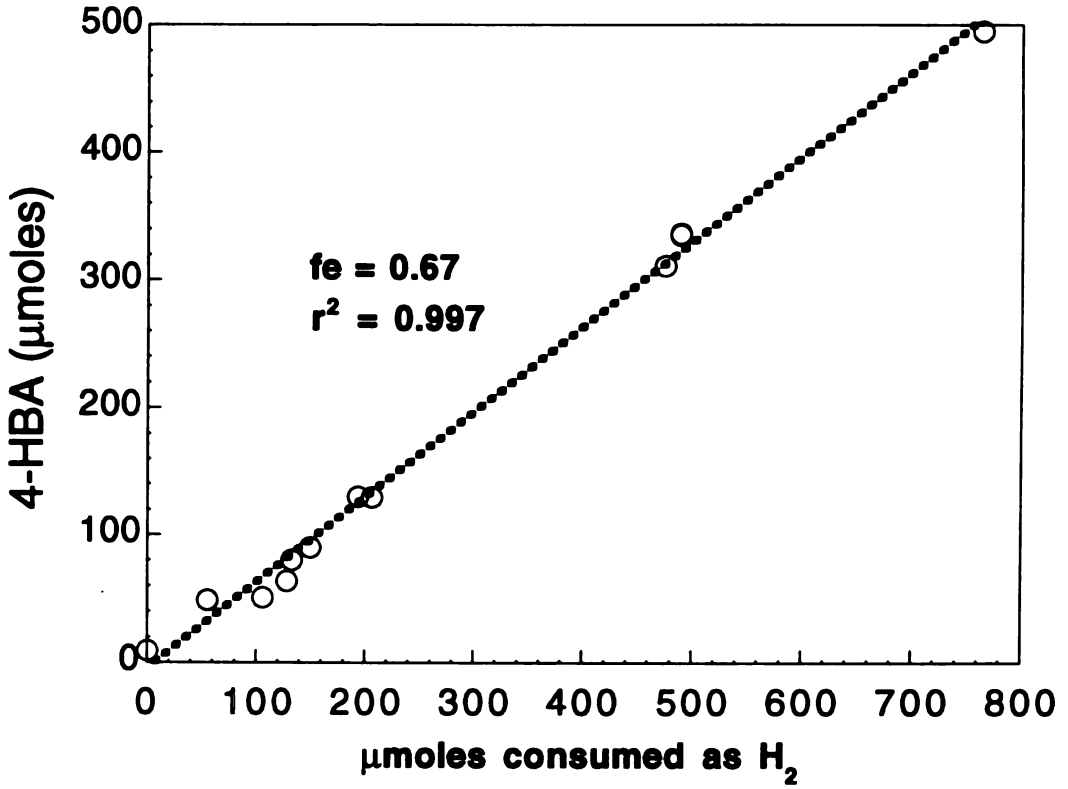

Figure 4.3. Graphical determination of **fe**, the fraction of electrons from the electron donor going to the electron acceptor. Electrons from lactate are plotted as µmole equivalents of H_2 generated in the incomplete oxidation to acetate.

Table 4.1. Mass balance of electron donors (lactate, formate and butyrate) and 3Cl-4-HBA consumed and yield of cells as a dry-weight.

	Concentration (mmoles)					Yield (g/mole substrate)		
Culture	H_2 from substrate equiv.	4-HBA	acetate	cells mg (mmole)		H_2 equiv.	3Cl-4-HBA	fs
Lactate A	2.94[a]	1.26	1.30	9 (0.080)[b]	} ave.	3.24 (0.22)[a]	6.90 (1.11)[a]	0.285[c]
Lactate B	2.84	1.27	1.34	9.8 (0.087)				
Lactate C	1.00	0.55	0.38	3.2 (0.028)				
Formate and Butyrate	1.65	1.16	0.61	2.8 (0.025)[b]		1.69	2.42	0.15

[a] Three replicates with yields shown as mean and 95% confidence interval in parenthesis.

[b] mmole equivalent of the cell mass based on a formula weight of 113, assuming biomass composition of $C_5H_7O_2N$.

[c] fs = fraction of electrons from electron donor incorporated into biomass.

Table 4.2. Electron donors tested for use by strain Co23 in a minimal medium. 2,3-DCP or 3-Cl-4-OH-benzoate served as electron acceptors. A positive score indicated growth which was monitored by measuring the depletion of electron donor and acceptor. Cultures were grown at 30° C.

Substrate(moles)	Growth		Product (moles per mole substrate)
	3Cl-4-HBA	2,3-DCP	
Acetate(1)	-	-	ND
Formate(1)	+[a]	+[b]	none
Succinate(1)	-	-	ND
Propionate(1)	-	-	ND
Butyrate(1)	+	+	acetate(2)
Crotonate	nd	+	acetate
H_2	+[a]	+[a]	none
Fumarate(1)	nd	-	ND
Pyruvate(1)	+[c]	nd	acetate(1.0)
Lactate(1)	+	nd	acetate(1.0)

[a] Growth occurred only if acetate was present as a source of carbon.
[b] Formate utilization observed in the presence of butyrate.
[c] Pyruvate supported growth both fermentatively and in the presence of an electron acceptor.
ND – not determined

consumption of both electron donor and acceptor over three successive feedings. All of the organic acid electron donors, except formate, were incompletely oxidized to acetate. Pyruvate also supported growth in the absence of an electron acceptor and was fermented stoichiometrically to acetate.

Growth of strain Co23 on lactate and several different electron acceptors, as measured optical density, is shown in Figure 4.4. No increase in turbidity occurred with lactate alone or with lactate plus sulfate. Sulfite, thiosulfate and sulfur all supported growth as electron acceptors, but sulfate, nitrate and fumarate did not. Several halogenated phenols supported growth of strain Co23 (Table 4.3). *Ortho*-substituted chlorines were removed from polychlorinated phenols with two or greater halogen substituents, with the exception of 2,4- and 2,5-DCP. Mono-substituted halogenated phenols were not utilized by strain Co23. 3Cl-4-HBA and 3-Cl-4-hydroxyphenyl-acetate were the only mono-chlorinated substrates used as electron acceptors.

Growth and dechlorination rates. Due to the difficulty of maintaining sufficient 3Cl-4-HBA in the culture medium to sustain exponential growth of strain Co23, growth rates were measured using pyruvate as the sole carbon and energy source. Additions of only 2 - 3 mM 3Cl-4-HBA were possible, since higher concentrations inhibited growth. Strain Co23 exhibited typical exponential growth with a doubling time (t_d) of 15.4 h when grown on pyruvate at 30° C (Figure 4.5). When tested at several temperatures, growth was observed from 15° to 37° C, with the optimal growth rate expressed as μ_m ($1/t_d$) equal to 0.17 h^{-1} at 37° C (Figure 4.6). No growth was observed above 45° C.

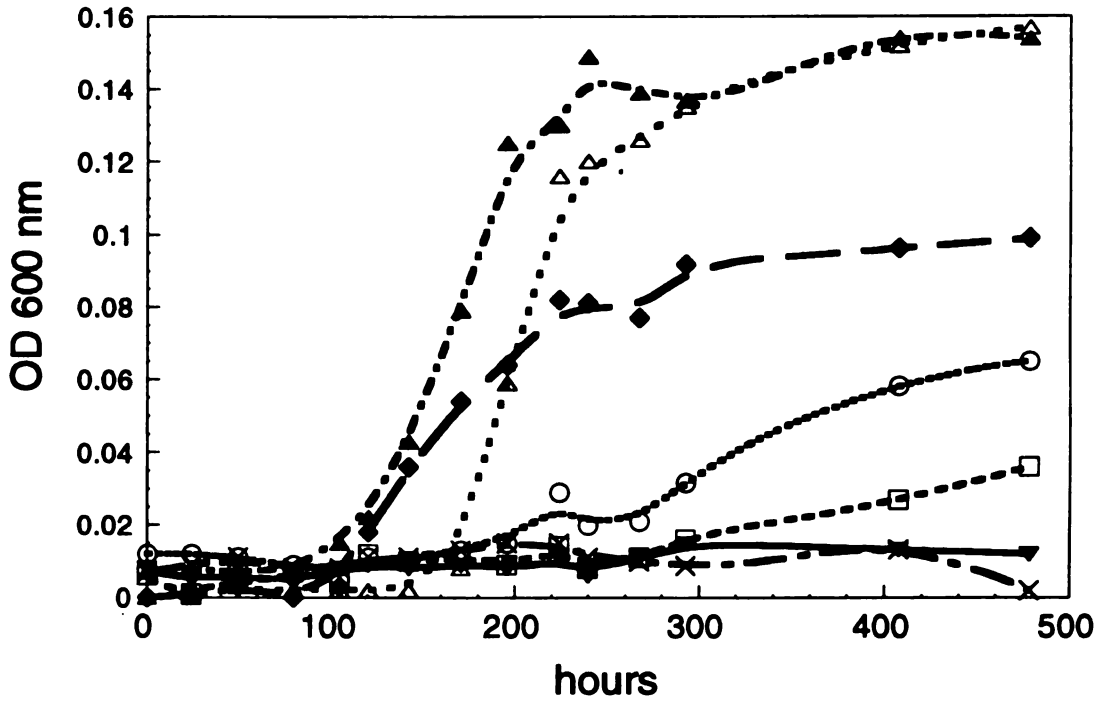

Figure 4.4. Growth of strain Co23 on different electron acceptors and lactate as measured by increases in turbidity (OD 600 nm). The symbols are as follows: sulfur (□), sulfite (Δ), thiosulfate (▲), sulfate (X), 3Cl-4-hydroxyphenylacetate (○), 3Cl-4-HBA (◆), and lactate (▼). Data are the average of duplicate cultures.

Table 4.3. Electron acceptors tested with strain Co23 with (+) indicating support of growth and (-) indicating no growth. Growth was determined by measuring the depletion of the electron donor and electron acceptor as well as observing visual increase in culture turbidity. Lactate or butyrate were used as electron donors. Cultures were incubated at 30° C.

Electron acceptor	Growth
2-CP	-
3-CP	-
4-CP	-
2-Fluorophenol	-
2-Bromophenol	-
2-Iodophenol	-
2,3-DCP (3-CP)[a]	+
2,4-DCP	-
2,5-DCP	-
2,6-DCP (2-CP)	+
2,4,6-TCP (4-CP)	+
2,4,6-Tribromophenol (4-BrP)	+
2,3,5-TCP	-
PCP	-
3Cl-4-HBA (4-HBA)	+
3-CBA	-
3-Cl -4-OH-phenyl-acetate (4-OH-pheny-acetate)	+
3-Cl-L-tyrosine	-
3-Cl-anisaldehyde	-
Other electron acceptors	
Sulfate	-
Sulfite	+
Thiosulfate	+
Sulfur	+
Nitrate	-
Fumarate	-

[a] Products of dechlorination are noted in parentheses.

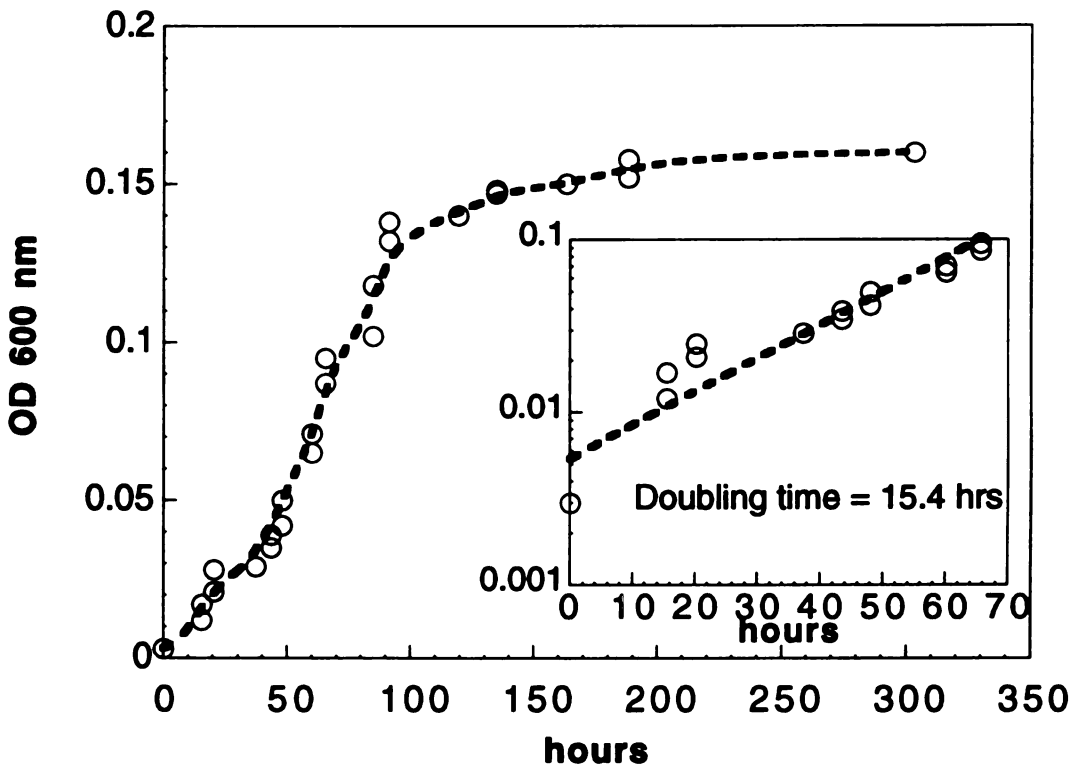

Figure 4.5. Exponential growth of strain Co23 on pyruvate at 30° C. Data are averaged from triplicate cultures.

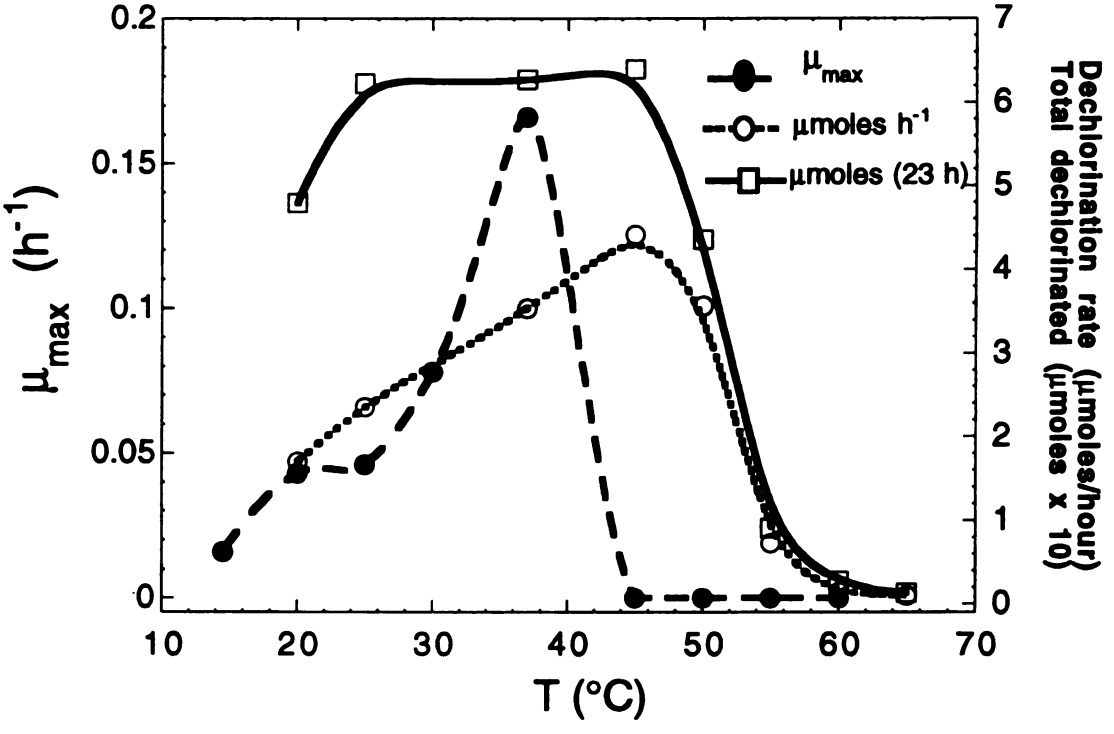

Figure 4.6. Temperature dependence for growth and dechlorination by strain Co23. Growth rates were determined by averaging the μ_{max} for duplicate cultures. Dechlorination rates were determined with resting cells grown on pyruvate at 37° C and incubated at various temperatures with 3Cl-4-HBA and pyruvate. Also shown are the μmoles of 3Cl-4-HBA dechlorinated over 23 h.

Dechlorination rates were determined for resting cells incubated at temperatures from 15°C to 65° C. Dechlorination activity had measureable rates at temperatures as high as 55° C and the maximal rate was observed at 45° C (Figure 4.6). Complete dechlorination of 4 mM 3Cl-4-HBA occurred within 23 h at temperatures ranging from 25° to 45° C (Figure 4.6). Although initial dechlorination rates at 50° C were significant, no further activity was observed after 11 h of incubation (data not shown). At a growth temperature of 30° C, resting cells dechlorinated 3Cl-4-HBA at a rate of 3.44 (+/- 1.09) mmol $h^{-1}g^{-1}$ cells dry weight.

Phylogeny of strain Co23. Comparison of available 16S rRNA sequences to that of strain Co23 indicated that strain Co23 is a member of the *Desulfotomaculum* group of the gram-positive bacteria (RDP release version 5). Pairwise similarity values calculated between Co23 and the available near complete 16S sequences from the *Desulfotomaculum* group produced similarity values ranging from 82.2% identity with *Peptococcus niger* to 97.2% identity with *Desulfitobacterium dehalogenans*, a bacterium that is also able to reductively dechlorinate chlorophenols (Table 4.4). Partial 16S sequences are avaliable for several other closely related bacteria. These sequences were included in the construction of a maximum likelihood phylogenetic tree (Figure 4.7). Bootstrap analysis of the maximum likelihood phylogenetic tree confirmed the close specific relationship between Co23 and *D. dehalogenans*.

Discussion

The most distinctive feature of strain Co23 is that it was able to couple growth to halorespiration. The term halorespiration has been suggested for those organisms that are capable of utilizing the considerable energy released from reductive dehalogenation for oxidative phosphorylation. The $\Delta G^{\circ\prime}$ for

Table 4.4. Pairwise similarity values for strain Co23 and selected bacterial species[a].

	1	2	3	4	5	6	7
1) *D. dehalogenans*	100						
2) *D. chlororespirans* str. Co23	97.2	100					
3) *P. niger*	82.6	82.2	100				
4) *H. chlorum*	87	86.3	82.6	100			
5) *Desulfotomaculum nigrificans*	84.9	84.2	82.8	85.0	100		
6) *B. subtilis*	83.7	83.7	80.8	82.4	83.1	100	
7) *E. coli*	78.8	78.9	76.8	78.8	77.7	78.8	100

[a] Similarity values were calculated for 1384 aligned residues. Species as in Figure 4.7.

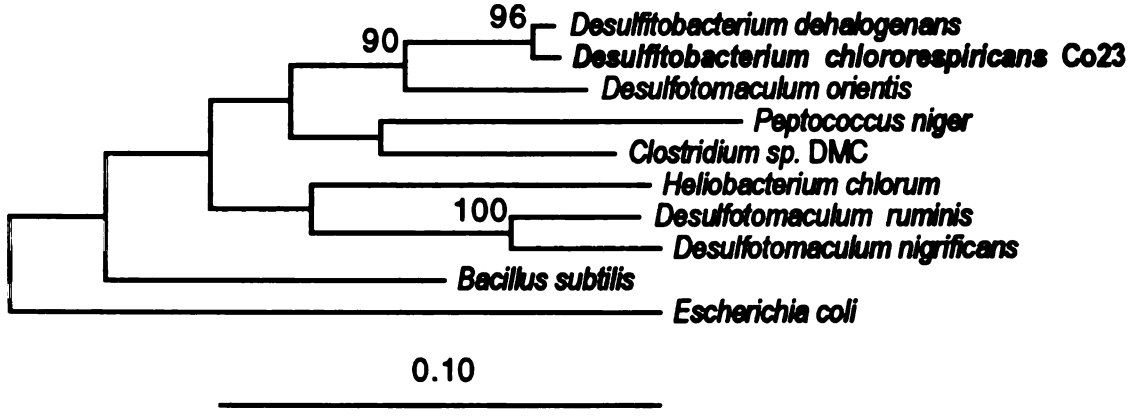

Figure 4.7. Maximum likelihood phylogenetic tree. Numbers at internal nodes are the percent of 100 bootstrap samples in which the group to the right of the node was monophyletic. Scale is in expected number of substitutions per positions.

reductively dechlorinating 2,3-DCP to 3-CP is -144.3 kJ/reaction (Dolfing and Harrison 1992). When coupled to the oxidation of lactate ($\Delta G^{o'}$ -2.1 kJ/mol H_2) or butyrate ($\Delta G^{o'}$ +24.1 kJ/mol H_2) the overall reaction is strongly exergonic (Thauer et al. 1977). Strain Co23 is one of only a few microorganisms described to grow by this process. The anaerobic myxobacterium strains 2CP-1, 2CP-2, 2CP-C and 2CP-3 were the first microorganisms described that are able to couple acetate oxidation to the reductive dechlorination of 2-chlorophenol (Cole et al. ; Cole et al. 1994). *Desulfitobacterium dehalogenans* may also grow via halorespiration, however yeast extract was always present in the medium and growth in the absence of this additive at the expense of reductive dechlorination has not yet been clearly established (Utkin et al. 1994).

The evidence for strain Co23's ability to grow with halogenated substrates as electron acceptors is as follows. Lactate was shown to support considerable growth with 3Cl-4-HBA as an electron acceptor, but no growth occurred when only lactate was present. Similar results occurred with butyrate and formate. The reducing equivalents from the partial oxidation of lactate to acetate are partitioned to 3Cl-4-HBA dechlorination and to biomass. The **fe** was determined to be 0.67, implying that 67 % of the available electrons from lactate are used for reductive dechlorination. From the cell yield data it was possible to calculate a value for **fs**, the fraction of electrons from lactate going to energy for cell synthesis (Table 4.1). Hence **fs** + **fe** equals 0.96, which is close to the theoretical optimum of 1.0. Therefore the data account for 96% of the electrons used from lactate oxidation with 67% going to electron acceptor reduction, which is consistent with the partitioning expected in respiratory processes (Criddle et al. 1991). Using both the **fe** and **fs** values the following

equation accounts for the mass balance of lactate oxidation, 3Cl-4-HBA reduction and cell yield:

0.25 lactate + 0.064 H_2O + 0.355 3Cl-4-HBA + 0.0145 NH_3 ---> 0.214 acetate + 0.25 CO_2 + 0.0145 $C_5H_7O_2N$ + 0.355 4-HBA + 0.355 HCl

Additional evidence for halorespiration is the measured H_2 concentration in cultures incubated for one week. Cord-Ruwisch et al. (1988) have shown that the steady-state concentration of hydrogen in a culture is largely dependent on the available energy associated with the reduction of the terminal electron acceptor. Thus the H_2 threshold concentration is when H_2 oxidation is no longer energetically favorable to the cell for the reduction of an electron acceptor. This is reflected in the "critical" free energy (ΔG_c), which is the minimum energy required that will permit growth (Seitz et al. 1990). Given this relationship, one would predict that threshold concentrations would decrease in the following order: H_2:CO_2 acetogenesis > methanogenesis> sulfidogenesis > fumarate reduction ≥ halorespiration ≥ NO_3^- reduction to NH_3 > Fe^{+3} reduction > denitrification > O_2 reduction. Our data show a H_2 threshold of less than 1 ppm, which is in good agreement with what is expected if halorespiration was the process responsible for H_2 consumption. Other halorespiring strains were shown to lower H_2 concentrations to 0.7 ppm and to less than 0.5 ppm for *Desulfomonile tiedjei* DCB-1 (DeWeerd et al. 1991) and 2CP strains (Cole et al.), respectively.

Although phylogenetically similar to *Desulfitobacterium dehalogenans* (Utkin et al. 1994) and physiologically similar to strain DCB-2 (Madsen and

Licht 1992), strain Co23 has several features that delineate it from these two microorganisms (Table 4.5). Pyruvate is fermented homoacetogenically by Co23 and DCB-2, in contrast to *D. dehalogenans* which produces considerable amounts of lactate (Utkin et al. 1994). Nitrate and fumarate support growth of *D. dehalogenans* and DCB-2, but do not support growth of strain Co23. Butyrate is used as an electron donor for dechlorination by strain Co23 in contrast to the case for*D. dehalogenans* . Although strain Co23 stains gram negative, the Desulfotomaculum group does not yield a reliable gram stain (Campbell and Singleton Jr. 1986). Utkin et al. (1994) demonstrated a gram-positive cell wall for *D. dehalogenans* by TEM and, since strain Co23 is phylogenetically closely related to *D. dehalogenans,* Co23 is likely to have the same type of cell wall structure. Neither *D. dehalogenans* nor DCB-2 have yet been shown to grow via halorespiration, since both were always cultured with considerable amounts of yeast extract. Strain Co23 is not able to utilize 2,4-DCP while the other two strains did, although cell-free assays have shown that the dehalogenating enzyme system does have some activity toward this compound (Löffler et al.). Also, the same cell-free assays showed that 3,5-DCP, a compound utilized by DCB-2 (Madsen and Licht 1992), is not dechlorinated by Co23. Finally the rates of dechlorination of 3Cl-4-HBA are more than an order of magnitude greater for strain Co23 than *D. dehalogenans* (Table 4.5) (Utkin et al. 1995).

Strain Co23's 16S rRNA sequence places it close to *Desulfitobacterium dehalogenans* , with a similarity of 97.2 % (Table 4.4). In addition, secondary structure features of the Co23 sequence are consistent with those of *D. dehalogenans.* This would at least place Co23 within the *Desulfitobacterium* genus, but the sequence differences and especially key phenotypic differences suggest that Co23 is a different species. Recently it has been shown that

Table 4.5. Comparison of strain Co23 with two other isolated dehalogenating strains. The data for *Desulfitobacterium dehalogenans* and strain DCB-2 are from Utkin et. al. (1994). and (Madsen and Licht, 1992) respectively.

Trait	Co23	*D. dehalogenans*	DCB-2
Gram stain	-	+	+
Spores	+	-	+
Motility	+	+	+
Halorespiration [a]	+	nd	nd
3Cl-4-HBA dechlorination rate (mmol h^{-1}g $cells^{-1}$)	3.44[b]	0.04[b]	nd
Grows readily on solid medium[c]	+	-	+
Pyruvate fermentation products	Acetate	Acetate lactate	Acetate
Electron donors for dechlorination			
Lactate	+	+	nd
Pyruvate	+	+	nd
H_2	+	+	nd
Formate	+	+	nd
Butyrate	+	-	nd
Yeast extract	nd	+	+
Electron acceptors			
2,4-DCP	-	+	+
3,5-DCP	-	-	+
NO_3^-	-	+	+
Fumarate	-	+	nd

[a] Growth observed with electron donor, lactate, and a halogenated phenol as an electron acceptor, with no addition of yeast extract as a supplemental carbon and energy source.

[b] Dehalogenation rate by resting cells of 3Cl-4-HBA is shown on a cell dry-weight basis. Rate data for *D. dehalogenans* is from (Utkin et. al, 1995).

[c] Colonies are easily propagated on anaerobic plate media with no special modifications to the medium or incubation conditions.

strains at 16S rRNA evolutionary distances of 2.5 to 3.0 (97 to 97.5% sequence identity) are unlikely to share 70% DNA:DNA homology and therefore are likely to be different species (Stackebrandt and Goebel 1994). The key physiological differences are the inability of strain Co23 to use fumarate and nitrate as electron acceptors and homoacetogenic growth on pyruvate, all important physiological and taxonomic traits for anaerobes. Although strain Co23 and *D. dehalogenans* share very similar chlorinated substrate use patterns, this feature seems insufficient to group these two strains into the same species since it may reflect only one biochemical character. A phylogenetic comparison with DCB-2 is not possible since the 16S rRNA sequence of this organism is not available, however this strain shares some physiological characteristics with Co23 that are not shared with *Desulfitobacterium dehalogenans* . For example DCB-2 was reported to grow homoacetogenically on pyruvate and produced visible spores (Madsen and Licht 1992). Although many features of comparison have not yet been measured for DCB-2, it is unique in its ability to dechlorinate 3,5-DCP to 3-CP.

The specificity that strain Co23 exhibits for removing *ortho*-substituted halogens from poly-halogenated phenols and the coupling to respiratory growth suggests that this capability has evolved for some period of time. One hypothesis is that natural halogenated compounds are ubiquitous and abundant enough to select for and support novel anaerobic microbial populations. A possible natural source of halogenated aromatic compounds are fungi. Recently a white rot fungus was reported to generate concentrations of up to 75 mg kg^{-1} of litter of chlorinated anisyl metabolites, eg. 3-chloroanisaldehyde (de Jong et al. 1994). Although strain Co23 could not directly use this compound, 3-Cl-anisaldehyde could reasonably undergo

demethylation and oxidation reactions to produce 3Cl-4-HBA, the best growth substrate for halorespiration by strain Co23. Another naturally occurring aromatic amino-acid, 3-chlorotyrosine (Gribble 1992), also was not dechlorinated by strain Co23. Again, however, it is possible that degradation of this compound in an anaerobic environment could result in 3Cl-4-HBA or 2-CP. The presence of either 3-chloroanisaldehyde or 3-chlorotyrosine in the original compost-soil microcosm, however, has not been established. Bromophenolic compounds are produced by marine hemichordates and have been used to enrich for an anaerobic debrominating microorganism (Steward et al. 1995). Several other naturally occurring chlorophenolic compounds that could potentially serve as substrates for halorespiring microorganisms are discussed by Gribble (1992).

Strain Co23 has some features desired for bioremediaton, such as relatively rapid growth, compared to previously isolated aryl-halorespirers. Since growth is selected by the chlorinated substrate, the process is easier to manage than if it were by cometabolism. Strain Co23 has also been shown to dechlorinate a broad range of halogenated substrates in cell-free assays, generally being able to remove most *ortho*-substituted chlorines from pentachlorophenol to dichlorophenol (Löffler et al.). The temperature stability of the dechlorination activity may also be exploited by designing treatment systems that operate at higher temperatures. For example at 45° C the dechlorination activity would be stable, uncoupled from growth and more rapid compared to 37° C. Also the spore-forming capability should provide for long term survival of inoculum in a habitat.

Strain Co23 is also a good candidate for basic studies on the mechanism of halorespiration because it has faster growth rates and higher cell yields than

Desulfomonile tiedje DCB-1 and the anaerobic myxobacterium 2CP-1 (Cole et al. 1994). Since it is a gram-positive microorganism it is an important model system for comparison to the dechlorination process in gram-negatives.

Description of *Desulfitobacterium chlororespirans* sp. nov. *Desulfitobacterium chlororespirans* (chlor.o.resp.ir.i.cans' M. L. part. adj.; *chloro* referring to the group VII element chlorine; fr. L *respirare* to blow, breathe; *chlororespirans* , breathing chlorine, referring to the characteristic of coupling oxidation of electron donors to reductive removal of chlorines from various chlorophenolic compounds via a respiratory process used for obtaining energy for growth). Cells are slightly curved motile rods 3 to 5 μm long and 0.5 to 0.7 μm wide. Terminally located spores appear in late growth and cultures are resistant to heat-treatment at 80° C. Cells stain gram negative, but phylogenetically this organism is within the gram-positive Desulfotomaculum group. Growth is obligately anaerobic and sulfite, thiosulfate, sulfur and *ortho*-substituted polychlorophenolic compounds are used as electron acceptors. 3-Chloro-4-hydroxybenzoate is the best substrate for halorespiration. Pyruvate, lactate, formate, butyrate and H_2 are used as electron donors. Cells grow by partial oxidation of carbon substrates to acetate coupled to the reductive dechlorination of *ortho*-substituted chlorophenolic compounds. Growth of cells on pyruvate alone is homoacetogenic. The optimal growth temperature is 37° C. The pH range for growth is 6.8 to 7.5. Colonies grown on R2A agar medium are white, round and smooth with a 1-2 mm diameter after one week of growth. This organism was isolated from a Michigan residential compost soil microcosms fed dichlorophenols and nitrate. The type strain is Co23.

Acknowledgments

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Chapter V

Meta-dechlorination of 3-chlorophenol and 2,3-dichlorophenol in methanogenic enrichment cultures: the effects of inhibitors and different electron donors

Introduction

Reductive dechlorination has been known to support respiratory metabolism in several microorganisms (Dolfing 1990; Mohn and Tiedje 1990; Holliger et al. 1993; Cole et al. 1994), however evidence suggests that this reaction is not energy yielding for all dechlorinating microorganisms. For example, polychlorinated biphenyls (PCBs) are reductively dechlorinated in enrichments, but the majority of reducing power from organic matter with a few exceptions flows to methane (Bedard and Quensen III 1995) and enhanced growth as measured by increased dechlorination activity is not apparent. Similar results have been obtained in anaerobic reactors fed with chlorophenols (Perkins et al. 1994; Juteau et al. 1995). Thus, it is probable that, despite the potential energetic advantage from using reductive dechlorination, some microbes carry out this transformation without capturing this energy. The cell, however, may still benefit by detoxification if the substrate is toxic.

Chlorophenolic compounds have been used extensively in several industries and there has been considerable effort to study the degradation of these compounds under aerobic and strictly anaerobic conditions (Häggblom 1992; Mohn and Tiedje 1992). Several polychlorinated phenols and monochlorophenols (CPs) are degraded under aerobic and anaerobic conditions (Boyd et al. 1983; Gibson and Suflita 1986; Hrudey et al. 1987;

Genthner et al. 1989; Genthner et al. 1989b; Hale et al. 1990; Zhang and Wiegel 1990; Madsen and Aamand 1992). In many of the anaerobic polychlorophenol degrading communities studied, 3-chlorophenol (3-CP) and other *meta*-substituted phenols accumulated. As a result there are few enrichment cultures described that show *meta*-dechlorination activity of chlorophenols, particularly 3-CP. In anaerobic microcosms we observed the complete dechlorination of 2,3-DCP (Chapter 2). Enrichment cultures derived from these microcosms were used to further study the *meta*-dechlorination activity and to further simplify the consortium with the goal of obtaining a pure culture.

The anaerobic microcosms were fed a mixture of dichlorophenols. They exhibited *ortho*- followed by *meta*-dechlorination of 2,3-dichlorophenol (DCP) (Chapter 2). The enrichment cultures derived from these microcosms completely dechlorinated and degraded 2,3-DCP or 3-CP to CH_4 and CO_2. The objective of this work was to further characterize the type of dechlorination expressed in these cultures and to determine whether methanogens were responsible for the dechlorination. In addition, individual electron donors were evaluated to determine which were optimal for dechlorination. Our results suggest that a eubacterial population is responsible for the fortuitous reductive dechlorination of 3-CP and that a mixture of electron donors was the best choice for efficienct chlorine removal. The dechlorination activity also appeared to be inducible.

Materials and Methods

Enrichment Cultures and growth conditions. Enrichment cultures were inoculated with anaerobic compost microcosms that had been fed nitrate and mixtures of dichlorophenols. These cultures exhibited the complete

dechlorination of 2,3-DCP (Chapter 2). The enrichment cultures were fed acetate (1 mM) and 2,3-DCP or 3-CP (125 μM). Those enrichment cultures that showed dechlorination activity by the removal of *meta* and/or *ortho* substituted chlorines were transferred to fresh medium with either 2,3-DCP or 3-CP (125 μM). A mixture of 1.25 mM formate , 0.25 mM succinate , 0.25 mM propionate and 0.25 mM butyrate (VFAs) were added as electron donors to this and all subsequent enrichment cultures. Cultures were incubated at 30° C.

Cultures were grown in 160 ml serum bottles with 100 ml of boiled degassed medium or in anaerobic culture tubes with 20 ml of medium and closed with butyl rubber stoppers. The mineral salts medium (Stevens et al. 1992) and vitamin solution (Wolin et al. 1963) was provided as described previously (Chapter 2).

Inhibition of *meta*-dechlorination. Inhibitors specific for methanogens or eubacteria were used to test which microbial population was responsible for the *meta*-dechlorination of 3-CP to phenol. Bromoethane-sulfonic acid (BESA 1 mM) a methanogen inhibitor or Vancomycin (150 mg/l), a eubacteria inhibitor, were added to triplicate 20 ml cultures containing the VFA mixture described above and 100 μM 3-CP. Controls consisted of triplicate tubes with no inhibitors added and triplicate tubes that were autoclaved. One ml (5% v/v) from the enrichment culture which exhibited complete dechlorination and degradation of 3-CP to CH_4 was used to inoculate the tubes. Samples were taken at approximately three-week intervals and analyzed by HPLC for 3-CP, phenol, benzoate and VFAs. Headspace gas was analyzed for CH_4, CO_2 and H_2 at the same time. Once 3-CP or the VFAs were depleted in the culture they were replenished at the original concentrations by transferring from degassed stock solutions to the medium with sterile needles

and syringes. Additional BESA (2 mM) was added as needed to inhibit methanogenesis if CH_4 was observed.

Electron donors for *meta*-dechlorination. Formate (2 mM), 1 mM succinate, 1 mM propionate, or 1 mM butyrate were added individually to sets of triplicate anaerobic culture tubes to determine which VFAs supported dechlorination. Culture conditions were as described for the inhibition experiment. Individual electron donors were replenished to the medium once they had been depleted. 3-CP, phenol, benzoate, VFAs, and headspace gas were monitored as previously described.

Induction of dechlorination. Parallel cultures were incubated with the VFA mixture or phenol, but in the absence of 3-CP. Once methanogenesis was well established, additions of 125 μM 3-CP were made to triplicate sets of tubes with and without 4 mM BESA. A 3-CP plus VFA mixture culture served as the positive control. The time between start of dechlorination in the already active control culture and the onset of dechlorination in the test cultures was defined as the induction period. Culture conditions and monitoring were as previously described.

Utilization of 2,3-DCP. The effects of inhibiting methanogenesis on enrichment cultures capable of both the *ortho*- and *meta*-dechlorination of 2,3-DCP were examined. Triplicate sets of tubes were incubated with the VFA mixture plus 125 μM 2,3-DCP. One set of culture tubes received BESA (1 mM). Cultures were replenished with substrate and monitored as in previous experiments.

Analytical procedures. Chlorophenols, dichlorophenols and aromatic products of dechlorination were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was a phosphoric acid (0.1 %) buffered methanol pumped at 1.5 ml/min using a gradient from 48% to

55% methanol. A Hibar RP-18 (10 μm) column was used. Eluted peaks were detected at 218 nm, 230 nm and 275 nm simultaneously on a UV multiwavelength detector. Samples (1 ml) from the enrichments were taken, made basic with 10 μl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 μm filters prior to HPLC analysis.

VFAs were analyzed using a modification of the method described by Guerrant et al. (1982). A Schimadzu HPLC with a BioRad Aminex HPX-87H ion exclusion column heated to 60°C and using 0.005 N H_2SO_4 as the eluent was used for the analyses. Previously filtered samples were acidified to 0.25 N H_2SO_4 by adding 100 μl of 2.5 N H_2SO_4 to 900 μl of sample. Eluent was pumped at 0.6 ml/min and detection of VFAs was at 210 nm by a UV detector.

The headspace of the incubation vessels was analyzed for CH_4, H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity (TCD) detector. Headspace pressure was normalized to atmospheric by venting with a needle prior to removing 0.3 ml of gas for injection into the GC.

Results

Characterization of reductive dechlorination activity. Enrichment cultures inoculated from the DCP compost microcosm and fed 3-CP and 2,3-CP showed complete degradation of the chlorophenols under methanogenic conditions. 2,3-DCP was dechlorinated to 3-CP within 10 to 12 days of the start of incubation. *Meta*-dechlorination of 3-CP did not occur until six to eight weeks after inoculation and was inhibited by the presence of 2,3-DCP. Phenol accumulated in cultures fed 2,3-DCP and 3-CP, but was eventually converted to benzoate which appeared transiently. No 4-hydroxybenzoate was detected. Methane and CO_2 were the final products

detected in these enrichment cultures. Cultures received at least three additions of 2,3-DCP or 3-CP before being transferred. The data suggest the pathway shown in Figure 5.1.

Effect of inhibitors on reductive dechlorination. Reductive dechlorination of 3-CP in the enrichment cultures exhibited a lag time of 21 days (Figure 5.2A). The presence of BESA had little effect on dechlorination activity, although initial rates appeared to be slower. Final amounts of 3-CP dechlorinated were similar in cultures with and without BESA. Vancomycin totally inhibited dechlorination activity (Figure 5.2A), but had little effect on methanogenesis (Figure 5.2B). Some methanogenesis occurred in the BESA inhibited culture, however this was stopped by amending cultures with more BESA (Figure 5.2B). The total methane evolved from the inhibited culture was approximately 25 % of that produced by the uninhibited culture.

Effect incubation time on *meta*-dechlorination lag period. Since dechlorination activity required a considerable lag period, experiments were done to determine the induction period of dechlorination in cultures incubated in the absence of 3-CP. Prior to the addition of 3-CP, enrichments fed VFAs or phenol were incubated for 57 days. All VFAs and phenol were depleted in these cultures with concomitant production of methane. At the end of the incubation period 3-CP was added to all cultures, including a positive control which had been fed 3-CP plus VFAs throughout the 57 day incubation period. BESA was added to one set of the VFA-fed cultures to inhibit further methanogenesis. *Meta*-dechlorination commenced within 7 days in the control 3-CP + VFA and incubated VFA-fed cultures (Figure 5.3). The phenol-incubated culture showed no dechlorination of 3-CP, even though phenol degradation activity continued (data not shown). No further methane production

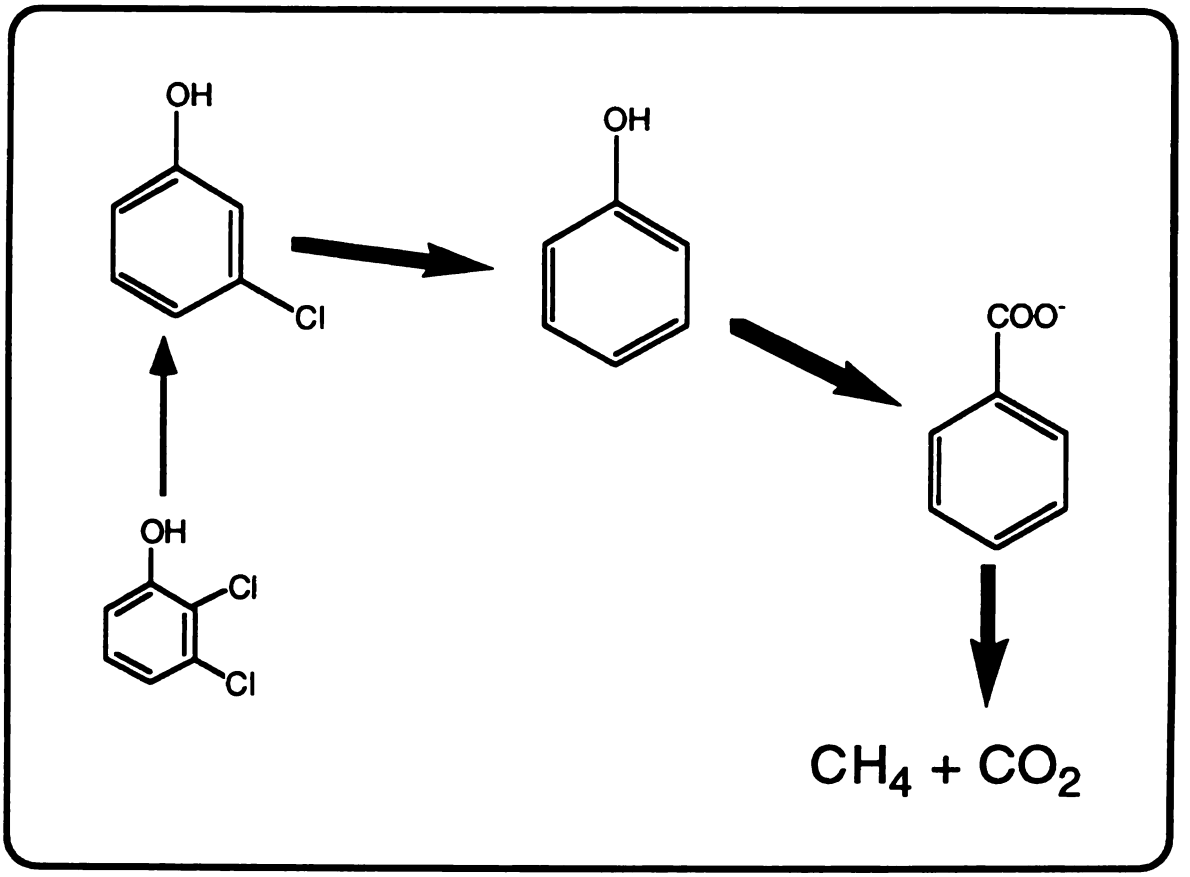

Figure 5.1. Schematic representation of the dechlorination pathway of 3-CP to phenol and subsequent tranformation to benzoate and methane. The *ortho*-dechlorination of 2,3-DCP is shown in the lower left and is converted completely to 3-CP prior to further degradation.

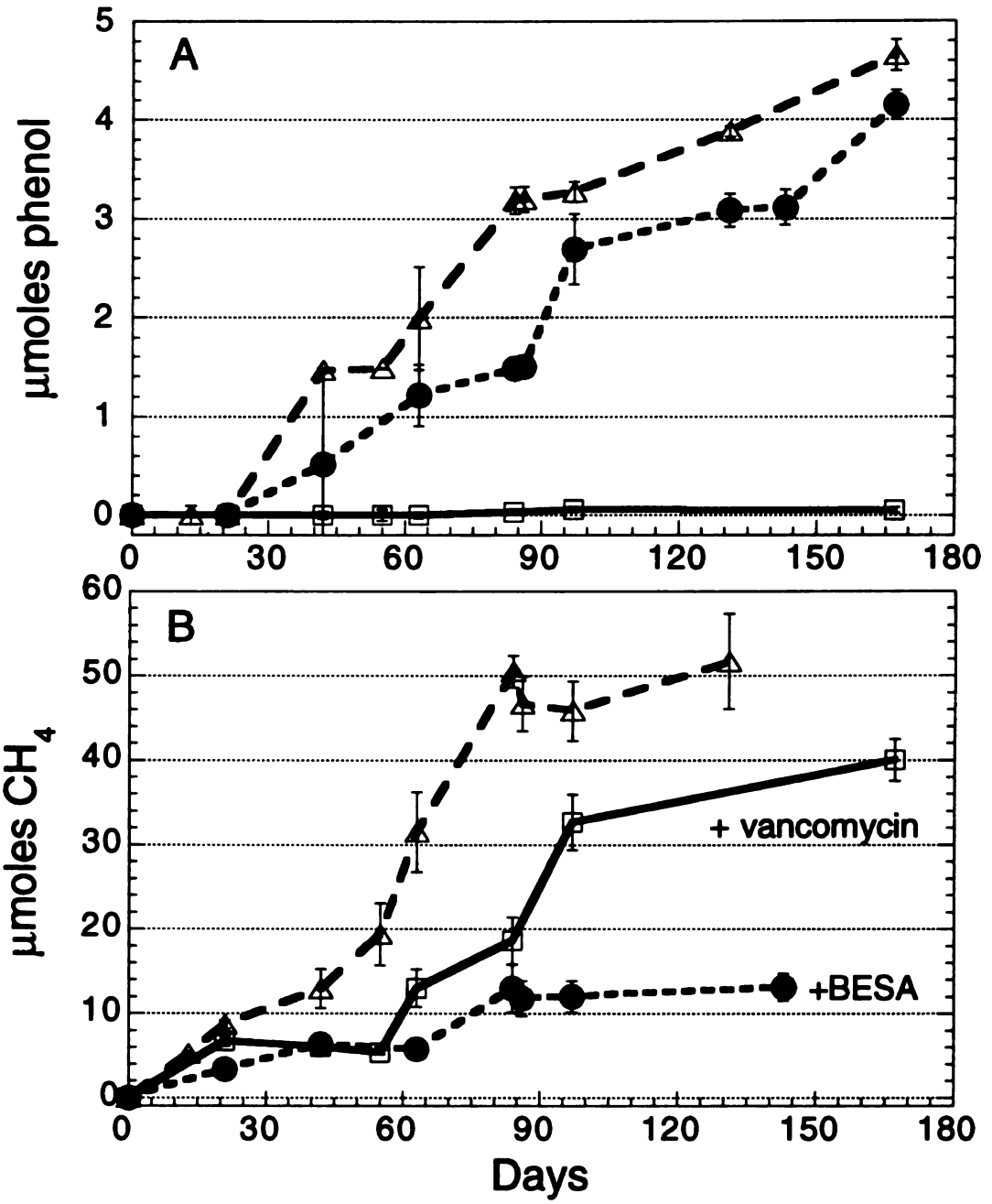

Figure 5.2. Effect of inhibitors on dechlorination in a methanogenic enrichment culture. (A) Cumulative 3-MCP dechlorination in cultures with VFA mixture (Δ), VFA plus BESA (●) and VFA plus vancomycin(□). (B) Methane production in the same cultures. Initial culture volume was 20 ml. Brackets are 95% confidence intervals.

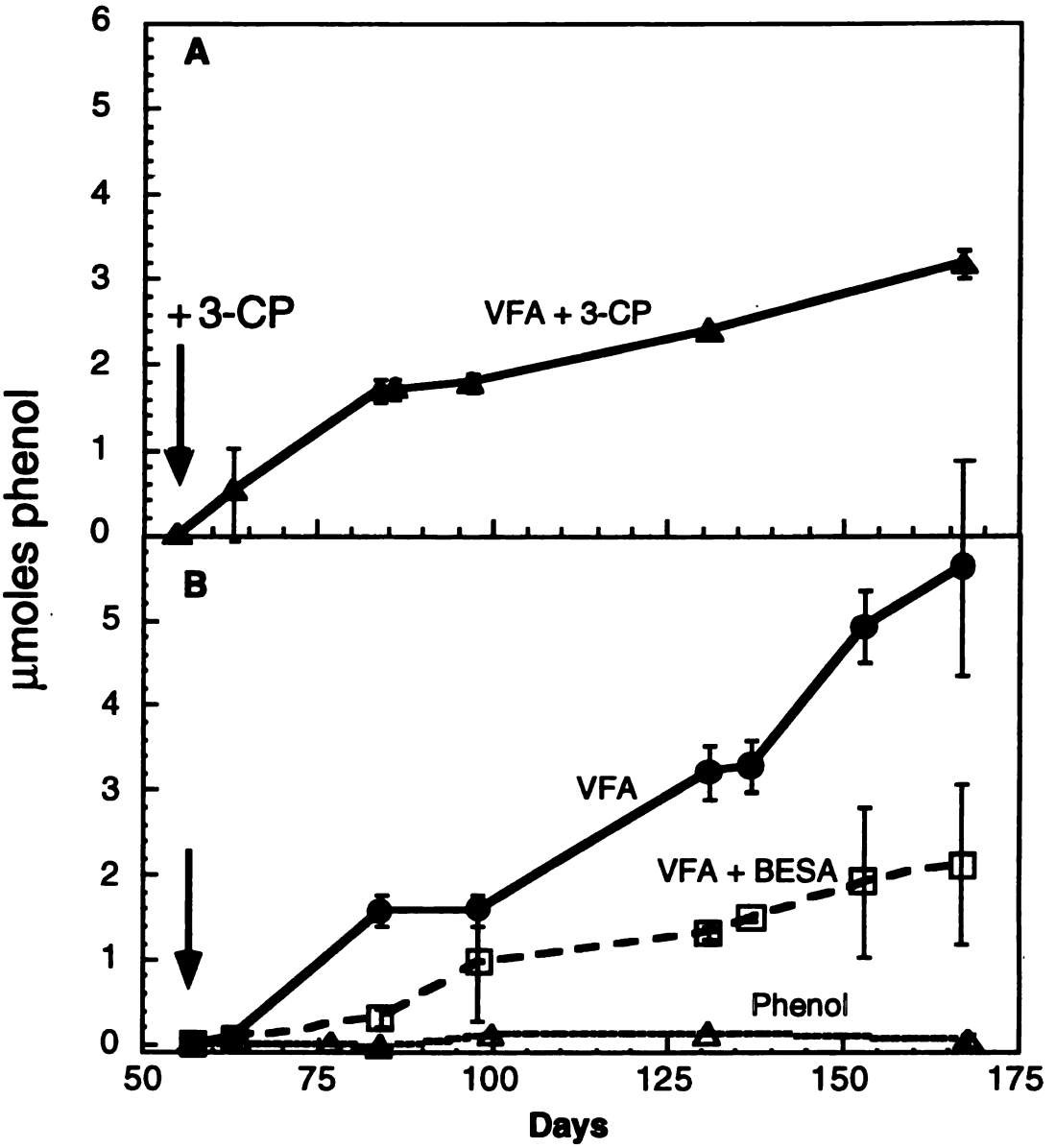

Figure 5.3. The dechlorination of 3-CP in cultures incubated with (A) and without (B) 3-CP for 57 days, and grown on VFAs. 3-CP with or without BESA was added at 57 days of incubation to VFA- and phenol-fed cultures. (A) shows the onset of dechlorination in the control culture grown on VFAs plus 3-CP upon the addition of 3-CP as designated by the arrow. (B) shows the onset of dechlorination in cultures incubated without 3-CP with the arrow indicating the addition of 3-CP and BESA. Brackets are the 95 % confidence intervals.

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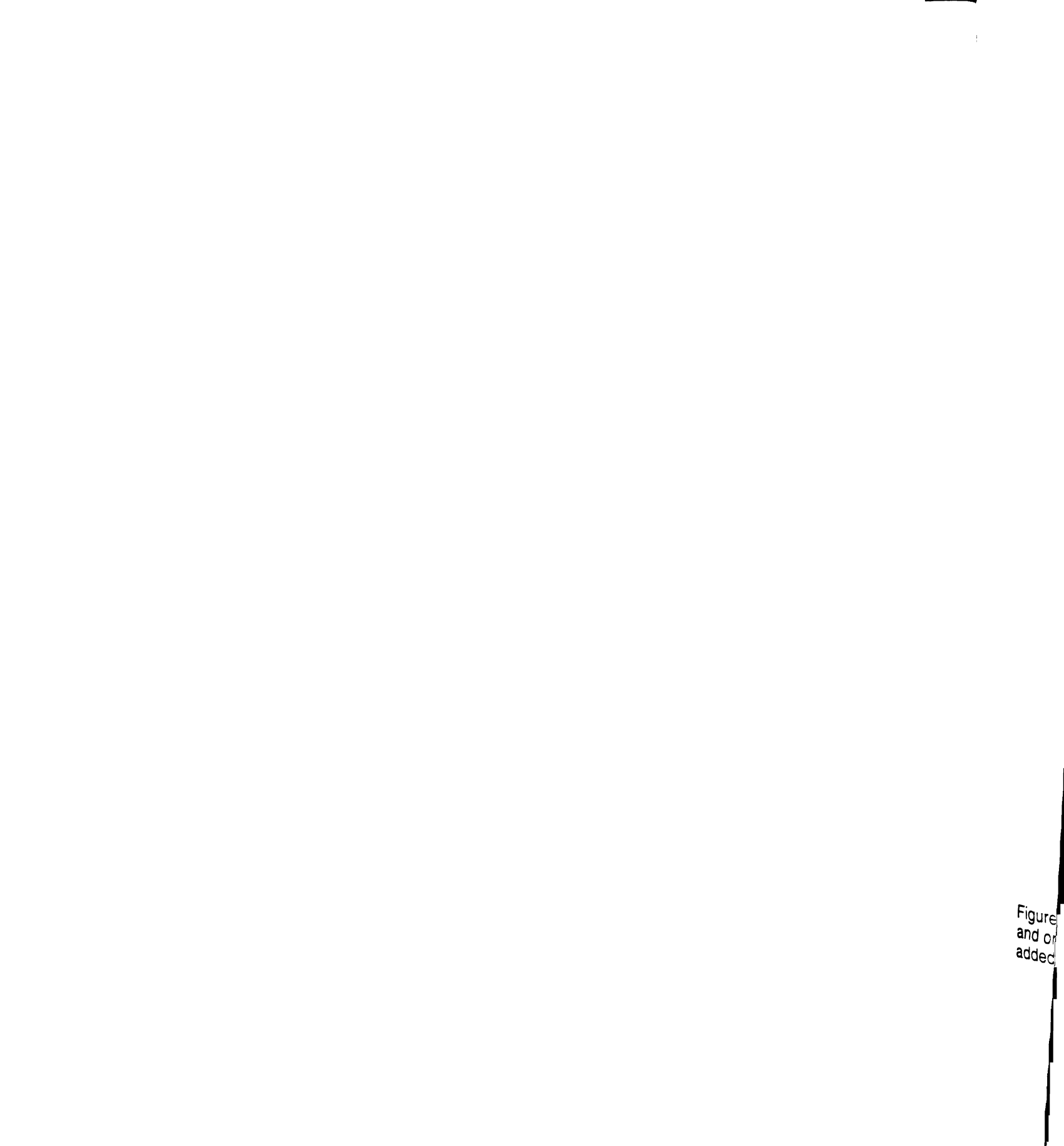
was observed in cultures with BESA and considerably less total dechlorination activity occurred compared to uninhibited cultures.

Dechlorination of 2,3-DCP. Methanogenic enrichment cultures that exhibited both *ortho*- and *meta*-dechlorination were treated with BESA to compare the effects of this inhibitor to those observed with the 3-CP cultures. Figure 5.4 A and B shows the *ortho*- and combined *ortho*- plus *meta*-dechlorination of 2,3-DCP in cultures without and with BESA. *Ortho*-dechlorination was not affected by the presence of BESA, as there was little variance in the rate or the extent of dechlorination with uninhibited cultures. Although there was apparently less total *ortho*- plus *meta*-dechlorination in the BESA-inhibited culture, the difference was not statistically significant. Considerable 3-CP remained in both treatments at the end of the experiment, and *meta*-dechlorination was never observed to occur simultaneously with *ortho*-dechlorination (data not shown). No methane production was observed in the inhibited culture, however considerable acetate accumulated in contrast to the uninhibited culture (data not shown).

Electron donors for *meta*-dechlorination. Formate, succinate, propionate and butyrate, as individual electron donors, supported reductive dechlorination of 3-CP. Enrichment cultures with formate or butyrate showed lag periods of less than 21 days before dechlorination occurred (Figure 5.5). For comparative purposes, the efficiency of dechlorination as mmoles dechlorinated per mole H_2 equivalent consumed of each electron donor was determined (Figure 5.5). At the end of the experiment the efficiency of dechlorination had reached a steady-state for each electron donor, with formate > propionate = succinate > butyrate. Early efficiencies were higher than at steady state for all electron donors, except formate. For example, at the start of



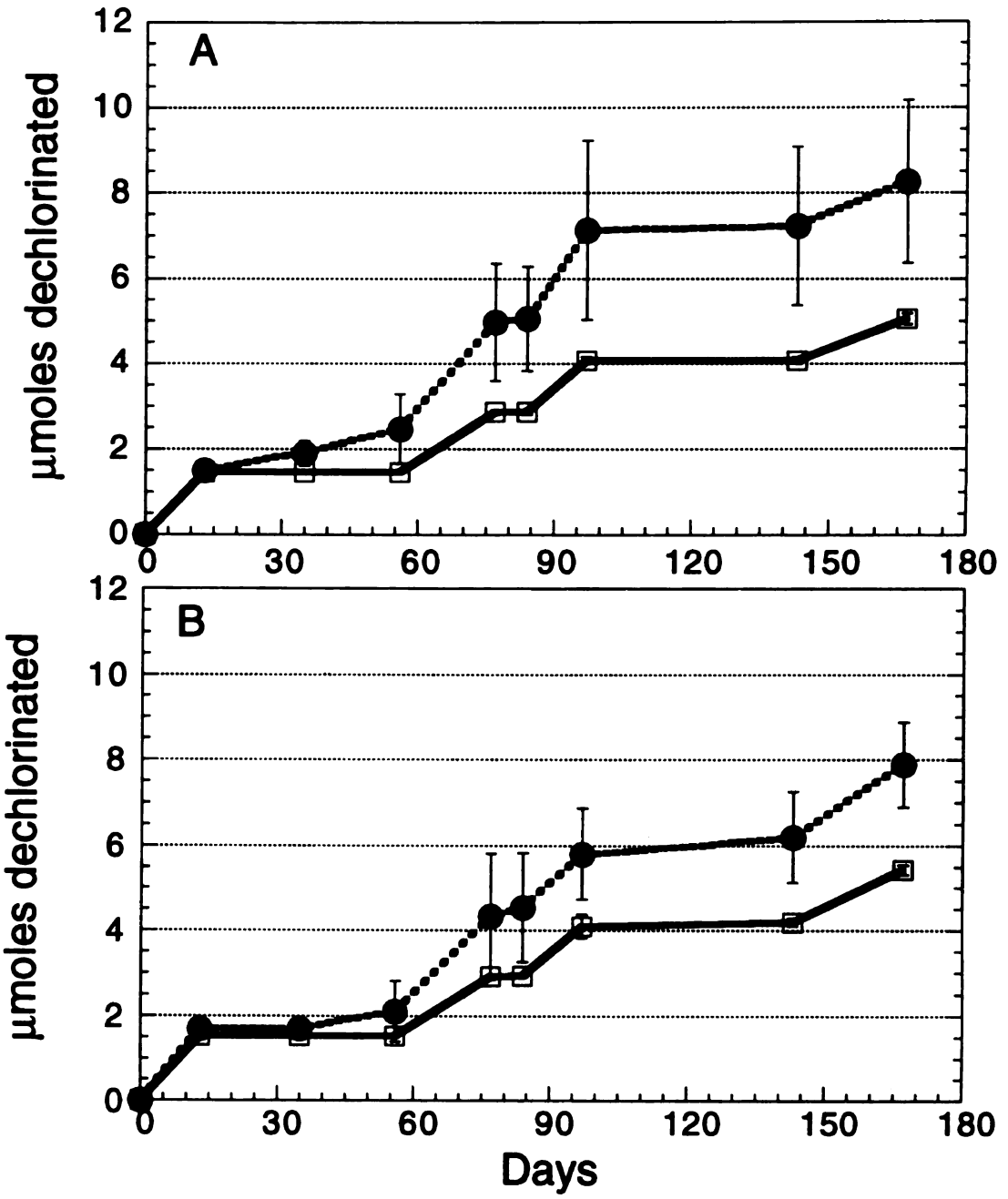


Figure 5.4. Cumulative dechlorination of 2,3-DCP. Ortho dechlorination (□) and ortho plus meta dechlorination (●). (A) has no BESA and (B) has BESA added. Brackets are 95% confidence intervals.

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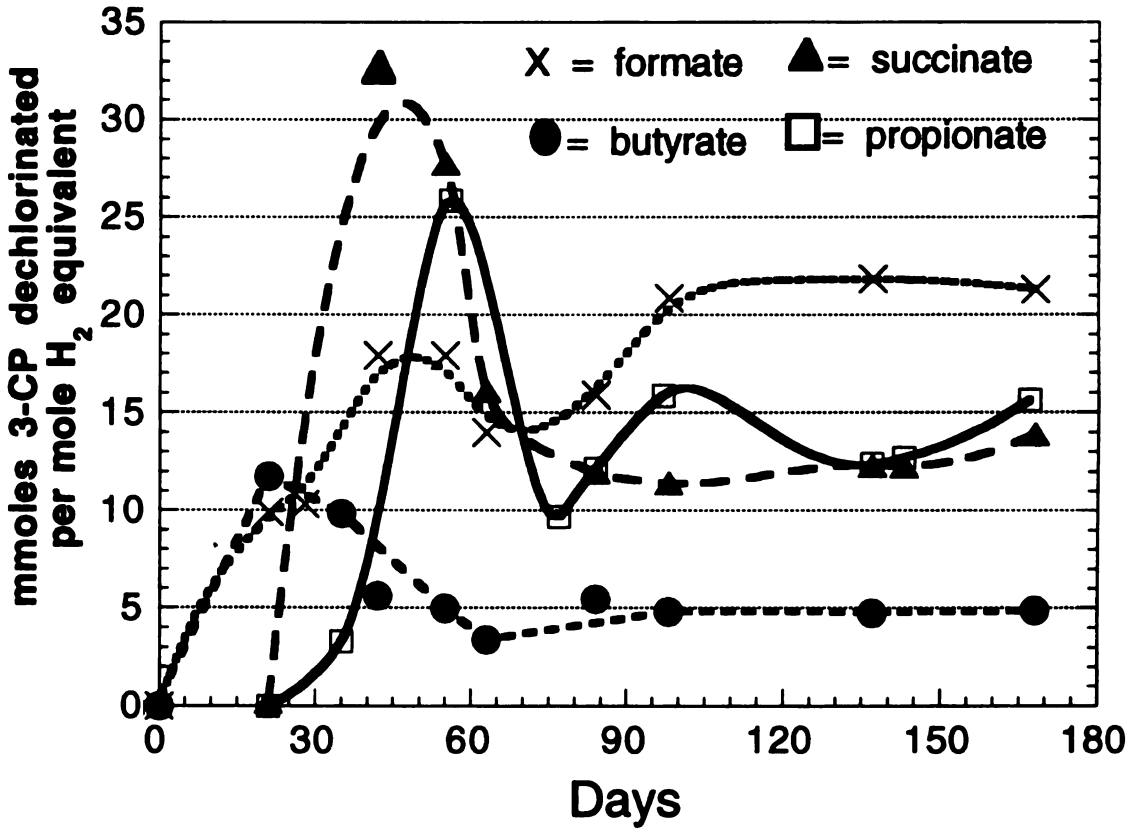

Figure 5.5. Effect of electron donor on the efficiency of reductive dechlorination of 3-CP as measured by the proportion of reducing equivalents used for dechlorination.

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dechlorination, propionate and succinate had nearly double the efficiency observed at the end of the experiment (Figure 5.5).

Data were compiled to calculate efficiencies of dechlorination for each treatment (Table 5.1). In addition, the molar yields of methane and acetate per mole H_2 equivalent of electron donor were calculated to provide reference indices for the different treatments. The most efficient dechlorination was observed in the 2,3-DCP + BESA treatment, which showed almost three times the value observed with any of the 3-CP treatments. *Ortho*-dechlorination accounted for an average of 67 % of the total dechlorination observed in the 2,3-DCP treatments. The enrichments provided a VFA mixture exhibited a dechlorination efficiency between that of succinate and formate alone, depending on the specific treatment.

The fraction of electrons from the electron donors used for reductive dechlorination was determined by calculating the slope of the linear regression line of the electron donor consumed as H_2 equivalents versus the equivalents dechlorinated. In pure cultures the fraction of electrons from the electron donor used to reduce the electron acceptor is referred to as the **fe**. When 3-CP was present, the apparent **fe** for *meta*-dechlorination was 0.01. With 2,3-DCP as the electron acceptor, the apparent **fe** was 0.05.

Discussion

Meta-dechlorination of chlorophenols has been observed in other anaerobic enrichments (Nicholson et al. 1992), but the types of microorganisms responsible for this activity have not been established. 3-CP often accumulates transiently in enrichment cultures, indicating that *meta*-dechlorination of this compound may not be common in the environment. Enrichment cultures derived from 2,3-DCP fed microcosms were shown to mediate *meta*-

Table 5.1. The effect of different electron acceptors and inhibitors on the efficiency of dechlorination, methanogenesis and the generation of acetate. The 95% confidence intervals are shown in parentheses.

Table 5.1. The effect of different electron acceptors and inhibitors on the efficiency of dechlorination, methanogenesis and the generation of acetate. The 95% confidence intervals are shown in parentheses.

Treatment	mmoles per mole H_2 equivalent				$\frac{CH_4 + acetate}{dechlorinated}$[a]
	Total dechlorinated[a]		CH_4	Acetate	
3- CP + VFAs[c]	13.0 (0.95)		187 (10.5)	0.61 (0.63)	14.4
3- CP + VFAs + BESA	22.1 (2.78)		103 (15.2)	165 (35.6)	12.1
3- CP + VFA s + vancomycin	0.46 (0.06)		250 (49.0)	25.6 (37.6)[d]	604
VFAs (+3-CP at 57 days)	16.8 (3.02)		92.2 (61.3)	1.20 (0.72)	14.7
VFA s (+3-CP + BESA at 57 days)	9.48 (0.81)		1.36 (2.30)	68.7 (14.0)	25.6
phenol (+ 3-CP at 77 days)	0.41 (0.25)		158 (12.6)	2.58 (2.59)	396
3- CP + formate	21.3 (0.55)		276 (27.1)	25.8 (25.1)	14.2
3- CP + succinate	12.5 (1.03)		180 (5.75)	1.22 (1.11)	14.6
3- CP + butryate	4.98 (0.31)		149 (12.5)	0.11 (0.22)	30.0
3- CP + propionate	14.2 (1.83)		165 (15.5)	0.44 (0.47)	11.7
2,3-DCP + VFA	29.2 (6.34)	61.6%[e]	166 (19.5)	5.14 (6.04)	5.9
2,3-DCP + VFA + BESA	51.7 (5.01)	73.1%[e]	3.96 (2.45)	52.5 (13.1)	1.1

[a] Ratio of electron donating efficiencies: mmoles methane plus acetate to mmoles dechlorinated.
[b] Meta-dechlorination of 3-MCP and ortho- + meta-dechlorination of 2,3-DCP.
[c] Mixture of formate, succinate, propionate and butyrate.
[d] Acetate was added to this culture as a supplemental electron donor.
[e] Percent of the total dechlorination due to the removal of ortho-substituted chlorines.

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dechlorination and thus were selected for further study. In the enrichment cultures vancomycin completely inhibits *meta*-dechlorination and BESA does not. This indicates that eubacteria are responsible for reductively dechlorinating the 3-CP to phenol in the enrichment cultures. Similar conclusions were reported for a methanogenic microbial community that dechlorinated 2,4,6-TCP (Perkins et al. 1994). Dechlorination, however, is not affected by methanogenesis, since uninhibited cultures have higher initial dechlorination rates and higher *meta*-dechlorination than BESA-inhibited cultures. Similar results are also found with 2,3-DCP fed cultures.

One interesting observation is that the incubation of the cultures on VFAs alone exhibits an equivalent lag period for dechlorination to the cultures incubated with 3-CP plus VFAs (Figure 5.3). This indicates that the microbial populations responsible for dechlorination are growing in the absence of 3-CP. It is also possible that the dehalogenation specific enzymes are induced in the absence of 3-CP, although it seems more likely that dechlorination is fortuitously mediated by a eubacterial population. The absence of dechlorination activity in the cultures fed phenol also supports this conclusion, since induction for specific dehalogenase activity might be expected with a similar compound like phenol. Since only the VFA mixture was provided as a electron donor source, there are only a limited number of energy yielding mechanisms possible for this methanogenic microbial community. The oxidation of each electron donor, except formate, would require a syntrophic interaction with a H_2 consuming methanogen or acetogen (Conrad et al. 1986; Schink 1988). Syntrophic microorganisms might benefit from reductive dechlorination of 3-CP by using it as a H_2 scavenging mechanism. An acetogen might also fill the H_2-scavenging niche, using the chlorophenol as an

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alternative sink for reducing equivalents and perhaps gaining energy from the process.

Experiments with individual electron donors show that all compounds tested could support *meta*-dechlorination activity, with formate being the most efficient. It is interesting that the VFA mixture exhibits similar efficiencies of dechlorination to formate. This indicates that the inclusion of formate as an electron donor may be sufficient to raise the efficiency of dechlorination. Formate is converted to H_2 and CO_2 in most anaerobic ecosystems and even methanogens that can use formate will first oxidize it to $H_2 + CO_2$. The H_2 from this reaction could then be used for methanogenesis with some also available to reductively dechlorinate 3-CP. It is possible that the microorganisms responsible for dechlorination require H_2 as an electron donor and therefore reactions that favor more H_2 generation would lead to more efficient microbial dechlorination. Since formate oxidation is not as energetically constrained, (i.e. requiring a syntrophic partnership, as would be the case for butyrate or propionate oxidation) there would be significant H_2 produced even in the absence of a methanogen (Chapter 7).

There is also the possibility that a homoacetogen is responsible for the dechlorination activity, since considerable acetate was observed in the formate-fed enrichment cultures. Acetate is also formed with the other electron donors although the mechanism of acetate generation differed in each culture. Butyrate is oxidized to two acetates. Propionate is converted to acetate and CO_2. Succinate is decarboxylated to propionate and subsequently to acetate and CO_2. Acetate appears transiently in the cultures and is converted into methane except in those cultures receiving BESA. Some H_2 is also formed from acetate during acetoclastic methanogenesis (Lovley and Ferrey 1985) and

therefore methanogens could be supplying reducing equivalents to dechlorinating microorganisms in the enrichment cultures.

The presence of both methanogenesis and acetogenesis in formate-fed enrichment cultures requires that H_2 concentrations are high enough to energetically support both of these activities. The threshold H_2 concentration, below which no energy can be obtained, is about 500 ppm for acetogens and 50 ppm for methanogens (Cord-Ruwisch et al. 1988). In order for both methanogens and acetogens to coexist, the H_2 concentration must be greater than 500 ppm. At this relatively high concentration of H_2 the dechlorinators may be able to act as scavengers of reducing equivalents, which they might not be able to do at lower H_2 concentrations. It is possible that the lower efficiency of dechlorination observed with butyrate is associated with a low H_2 concentration in that particular set of enrichment cultures. This may be due to the obligate syntrophic life-style of the butyrate oxidizers, which require low H_2 concentrations for growth (Schink and Friedrich 1994). In contrast, formate oxidation would release the equivalent amount of energy to butyrate at a higher threshold concentration of H_2 and therefore may support both acetogenic and methanogenic populations.

When the efficiencies of dechlorination are compared with the mmoles of methane produced per mole of reducing equivalent (Table 5.1), it is apparent that most of the reducing power in the enrichment cultures is not diverted to 3-CP reduction. Even when BESA was added to inhibit methanogenesis, the additional reducing equivalents end up in acetate rather than being used for reductive *meta*-dechlorination. An exception was observed with 2,3-DCP + BESA enrichment cultures, which have equal reducing equivalents routed to dechlorination and acetate production. This may be due to a microbial population in the enrichment culture using the energy from the *ortho*-

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dechlorination of 2,3-DCP. Some evidence supporting this assertion was found in strain Co23 isolated from this enrichment, which was able to grow using 2,3-DCP as an electron acceptor (Chapter 4). The amount of acetate serving as an electron sink is likely to be underestimated, since acetate could serve as a carbon source even in the presence of BESA. Therefore, it is possible that acetogenesis efficiencies in the different treatments were close to methanogenesis efficiencis. By calculating the ratio of CH_4 + acetate efficiencies to the dechlorination efficiency (Table 5.1), it is apparent that the best *meta*-dechlorination of 3-CP occurred in enrichments with ratios between 12 and 15. This is valid for several different treatments and suggests that both acetogenesis and methanogenesis may be important for *meta*-dechlorination even though a methanogen is not directly responsible for the dechlorination activity.

Since the *meta*-dechlorination activity is closely correlated to methanogenesis and acetogenesis activity in the enrichment cultures, it may be difficult to isolate the responsible microorganism. This is likely since the dechlorination of 3-CP did not serve as a major electron sink and therefore was not likely to act as a significant electron acceptor for a microorganism. The presence of 3-CP in the enrichment culture therefore offers no selective advantage to any microbial population beyond tolerance to its toxicity. Although not very specific, one strategy would be to isolate as many component populations as possible and screen them for the ability to mediate this reductive dechlorination reaction. Even this may not be successful, since the reaction may rely on the synergistic relationship between many microbial populations within the community. It would probably not be practical to test all possible combinations of isolates recovered. The desired microbial population could also be non-culturable under the conditions being used for growth. Perhaps the

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best strategy is to characterize the community by analyzing the 16S rRNA phylogeny of the component microbial populations, as has been done in several studies of difficult to isolate cultures (Ward et al. 1990; Schmidt et al. 1991). Knowing the phylogeny of the component populations could provide information for developing isolation strategies.

Results from these enrichment culture experiments suggest that certain combinations of electron donors may be better suited for stimulating *meta*-dechlorination of chlorophenols. This information would be useful for developing bioremediation strategies for chlorophenolic waste material. It appears that a mixture of VFAs containing formate is best suited for obtaining the most efficient dechlorination. In contrast the use of butyrate resulted in the lowest dechlorination efficiency and phenol showed no capacity to support reductive dechlorination. It is possible that the intermittant supply of formate to the culture was responsible for keeping the H_2 concentrations above the threshold concentration for methanogenesis and therefore keeping sufficient reducing equivalents available for reductive dechlorination. This suggests that the best strategy for stimulating the anaerobic dechlorination of chlorophenols is to use an intermittent feeding of formate and perhaps other electron donors.

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Chapter VI

Evidence for halorespiration in an anaerobic enrichment culture that simultaneously dechlorinates 2- and 3-chlorophenol to phenol

Introduction

Reductive dechlorination of chlorophenols has been observed in many anaerobic microbial studies (Mohn and Tiedje 1992). The ability to couple the energy released from this reaction to growth has only been verified in a few microorganisms (Dolfing 1990; Mohn and Tiedje 1990; Cole et al. 1994; Sanford et al. 1996). Since this reductive dechlorination reaction releases considerable energy (Dolfing and Harrison 1992), the ability to halorespire in anaerobic environments is potentially beneficial to the organism catalyzing this transformation. The types of natural halogenated aromatic compounds that might be present in the environment and support halorespiration are many (Gribble 1992), and recently de Jong and coworkers (1994) found that white rot fungi actually generate up to 70 mg/kg of chlorinated anisyl metabolites in leaf litter. Thus, not only is a halorespiratory metabolism potentially useful, it is likely to occur in natural anaerobic ecosystems.

The reductive removal of chlorines from aromatic compounds may be important to anaerobic bioremediation strategies where considerable contamination with chlorophenolic compounds has occurred. Once chlorines are removed, the resulting aromatic compounds are generally more amenable to oxidative attack. This was observed in denitrifying microcosms in which 2-chlorophenol (2-CP) was dechlorinated by one microbial population to phenol,

which was then degraded by denitrifiers (Chapter 2). Often it is shown that dechlorination of multi-chlorinated phenols is incomplete, with *meta*- and *para*-substituted chlorophenols accumulating in anaerobic cultures (Juteau et al. 1995). Many studies have shown the reductive dechlorination of PCP to di- and mono-chlorophenols (Nicholson et al. 1992). In most cases the order of dechlorination is *ortho*, *para* and finally *meta*, although the latter two have been known occur in the reverse order (Mikesell and Boyd 1986) (Chapter 2). These lesser-halogenated products of dechlorination are often more easily degraded under aerobic environmental conditions.

In this study an enrichment culture with the novel ability to simultaneously reductively dechlorinate *ortho*- and *meta*- chlorines from monochlorophenols and 2,5-DCP was characterized. *Ortho*-dechlorination of chlorophenols is common in anaerobic environments, however, *meta*-dechlorination is relatively rare and therefore its occurrence in an enrichment culture warranted further study. The measurement of H_2 threshold concentrations and the determination of the fraction of electrons from the electron donor used for reductive dechlorination, **fe**, suggested a halorespiring microbial population was responsible for the dechlorination activity. This is the first evidence presented that shows that *meta*-dechlorination of any chlorophenol could serve as a terminal electron acceptor for growth.

Materials and Methods

Enrichment Cultures and growth conditions. Enrichment cultures were inoculated from anaerobic soil microcosms that had been fed nitrate and mixtures of monochlorophenols (CP) and exhibited the complete dechlorination of 2- and 3-CP (Chapter 2). The enrichment cultures were fed acetate (1 mM) and 2-CP and 3-MCP (125 μM each). Those enrichment cultures that showed dechlorination activity by the removal of *meta* and *ortho*

substituted chlorines were transferred to fresh medium with either 2,5-DCP or the 2- plus 3-CP mixture (125 μM). A mixture of 1.25 mM formate , 0.25 mM succinate , 0.25 mM propionate and 0.25 mM butyrate (VFAs) was added as electron donors to these enrichment cultures. Subsequent transfers contained 10 mM formate or H_2 (5 % v/v) added to the headspace as the electron donors for dechlorination. Acetate (0.5 - 1.0 mM) was added as a carbon source. Chlorophenols and phenol concentrations were monitored by HPLC. Depletion of VFAs was also monitored by HPLC. The composition of headspace gases was evaluated by GC. The growth medium and conditions were as described in Chapter 2 of this thesis. Cultures were incubated at 30° C.

Determination of electron donors and acceptors. The ability of the enrichment culture to reductively dechlorinate 2- and 3-CP with different electron donors was determined in duplicate 20 ml culture tubes incubated at 30° C. Acetate, formate, butyrate, succinate, propionate, fumarate, lactate, pyruvate, crotonate and H_2 were tested separately as potential electron donors. The soluble VFAs were added to duplicate tubes to achieve concentrations of 0.5 or 2.0 mM. 18 μmoles (6.6 % v/v) H_2 was added to the headspace of the duplicate culture tubes. 2- and 3-CP (100 μM each) were added as electron acceptors. A 5 % inoculum was added to each tube from a VFAs and 2- plus 3-CP grown culture. Chlorophenols were replenished three times at concentrations of 100 μM to those cultures exhibiting dechlorination activity. Dechlorination and depletion of electron donor were used as an indication of a positive test for growth along with the ability to serially transfer the dechlorination activity in the culture.

The range of electron acceptors used by this enrichment culture was determined using the same growth conditions as for the electron donor determination. With the four VFA mixture serving as electron donors, 100 μM of

2-CP; 3-CP; 4-CP; 2,3-DCP; 2,4-DCP; 2,5-DCP; 2,6-DCP; 3Cl-4-HBA; o-fluorophenol; o-bromophenol; o-iodophenol; 2,3,5-TCP; 2,4,6-TCP; pentachlorophenol (PCP); and 3-chloroanisaldehyde were tested separately as halogenated electron acceptors. Nitrate, and fumarate were also tested at a concentration of 5 mM. Tubes were inoculated with 5 % inoculum from cultures grown on VFAs and 2- plus 3-CP. Halogenated substrates were monitored by HPLC and were replenished once depleted. Nitrate and fumarate concentrations were measured by HPLC. Consumption of VFAs was monitored by HPLC to verify physiological activity.

Hydrogen uptake and threshold determination. Hydrogen uptake was monitored in relation to dechlorination activity to test if the hydrogenase activity was closely linked to the terminal electron accepting capacity of the 2CP strains. Hydrogen (5.0 %) was added to the headspace of 100 ml cultures in 160 ml serum bottles. 2,5-DCP (100 μM) was added to these cultures and the H_2 concentration was monitored by GC as dechlorination occurred. After the complete dechlorination of the 2,5-DCP, an additional amendment of 100 μM was made. To determine the threshold concentration for hydrogen maintained by the enrichment cultures while dechlorinating, duplicate cultures were monitored for H_2 depletion with excess 2,5-DCP present. Threshold concentrations of H_2 were measured using a GC equipped with a reduction gas detector from Trace Analytical, Menlo Park, CA.

The ability of the dechlorinating enrichment culture to produce H_2 and dechlorinate was tested by inoculating a culture with 20 mM formate as the sole energy source and 100 μM 2,5-DCP. Acetate (1 mM) was added as a carbon source. A 3% inoculum from the eighth serial transfer maintained on $H_2 + CO_2$ and chlorophenols was used. These serum bottle cultures were incubated on a

shaker at 30° C. H_2 was monitored by GC and dechlorination of 2,5-DCP was monitored by HPLC. Formate depletion was checked by HPLC analysis.

Determination of fe. As an indication of energetic efficiency and that halorespiration was occurring, the fraction of electrons (**fe**) from H_2 used for the reductive dechlorination of 2- and 3-CP or 2,5-DCP was determined. Several replicates of the enrichment culture were grown on 5 % H_2, 7% CO_2, 1 mM acetate and 2,5-DCP (125 µM). Alternatively 2- and 3-CP (100 µM each) were used as electron acceptors. Control cultures received H_2 or 2,5-DCP alone. Concentrations of chlorophenols were monitored by HPLC and amendments of new substrate (125 µM) were made prior to the concentration reaching zero. Acetate was determined by HPLC. The **fe** was calculated by plotting the H_2 equivalents used versus the total µmoles dechlorinated and determining the slope of the regression line. For example the total µmoles Cl^- dechlorinated from 2,5-DCP was equal to the µmoles of phenol formed (X2) plus the µmoles of 2-CP and 3-CP present in the medium at a particular time point.

Analytical procedures. Chlorophenols, dichlorophenols and aromatic products of dechlorination were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was a phosphoric acid (0.1 %) buffered methanol pumped at 1.5 ml/min using a gradient from 48% to 55% methanol. A Hibar RP-18 (10 µm) column was used. Detection of eluted peaks was done at 218 nm, 230 nm and 275 nm simultaneously on a UV multiwavelength detector. Samples (1 ml) from the enrichments were taken, made basic with 10 µl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 µm filters prior to HPLC analysis.

Volatile fatty acids were analyzed using the Schimadzu HPLC with a BioRad Aminex HPX-87H ion exclusion column heated to 60°C and using 0.005 N H_2SO_4 as the eluent. Previously filtered samples were acidified to

0.25 N H_2SO_4 by adding 100 µl of 2.5 N H_2SO_4 to 900 µl of sample. Eluent was pumped at 0.6 ml/min and detection of VFAs was at 210 nm by a UV detector.

The headspace of the incubation vessels was analyzed for CH_4, H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity (TCD) detector. Headspace pressure was normalized to atmospheric by venting with a needle prior to removing 0.3 ml of gas for injection into the GC. N_2O was quantified on a Perkin Elmer 910 GC with a Porapak Q column and ^{63}Ni -ECD detector. Denitrification products were measured in an Ar headspace.

Nitrate was analyzed by HPLC using a Whatman Partisil 10 SAX column on a Schimadzu HPLC. The eluent was a 50 mM phosphate buffer (pH = 3.0) pumped at a rate of 1 ml/min. UV adsorption at 210 nm was used for detection. Samples from primary enrichments were diluted 1:100 in deionized H_2O prior to nitrate and nitrite analysis. A 40 µl sample was injected by an Alcott 738 autosampler.

Results

Enrichment culture activity. Enrichment cultures fed the VFA mixture and monochlorophenols showed complete dechlorination of 2- and 3-CP to phenol. As H_2 was observed in the headspace of these initial enrichment cultures, subsequent transfers were made to media containing either H_2 or formate as electron donors. Cultures maintained with H_2 as an electron donor exhibited simultaneous *ortho*- and *meta*-dechlorination activity with the stoichiometric production of phenol (Figure 6.1). These enrichments are subsequently referred to as OM, for *ortho* plus *meta* dechlorinating enrichment culture. Hydrogen uptake was correlated to the appearance of phenol in these

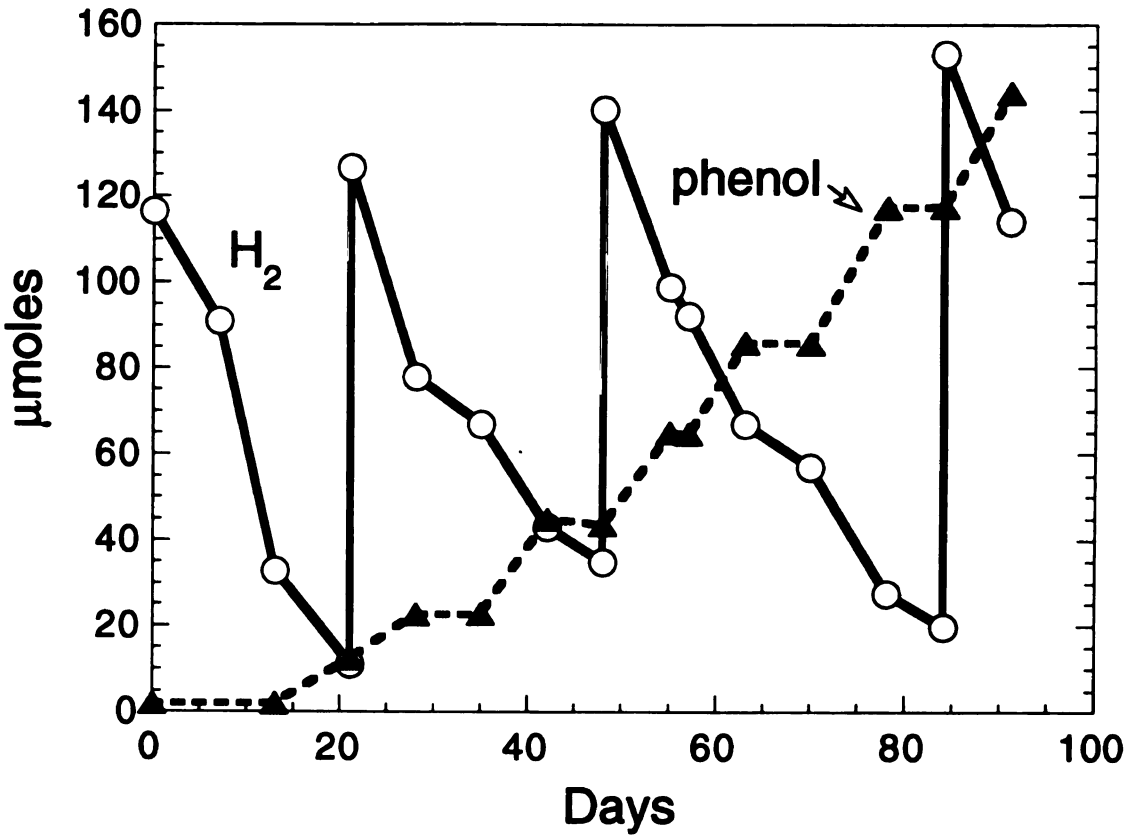

Figure 6.1. Uptake of H_2 by OM enrichment culture and cumulative production of phenol from the dechlorination of 2- and 3-CP.

enrichment cultures, although initial consumption of several μmoles of H_2 occurred prior to the onset of dechlorination activity. Control cultures containing only H_2 or only chlorophenols did not exhibit any H_2 uptake or dechlorination activity, respectively (data not shown).

Since 2- and 3-CP were used as electron acceptors, 2,5-DCP was used as an alternative substrate for dechlorination. 2,5-DCP was stoichiometrically dechlorinated to phenol in the presence of H_2 (Figure 6.2A). The transient appearance of 2- and 3-CP indicated that both *ortho*- and *meta*-chlorines were removed from 2,5-DCP sequentially with either position removed prior to the other (Figure 6.2B). The H_2 consumed is shown in Figure 6.2B. On day 110 of incubation, H_2 additions were suspended to determine the effects of no H_2 on dechlorination and to determine the threshold concentration of H_2 (see below). As the H_2 concentration was reduced, the rate of 2,5-DCP dechlorination slowed with 2- and 3-CP accumulating in the medium (Figure 6.2). This was confirmatory evidence of the requirement for H_2 for reductive dechlorination activity.

Electron acceptors and donors. Several chlorinated phenolic compounds were tested for dechlorination by the OM enrichment culture (Table 6.1). 3-CP was only dechlorinated when 2-CP was also present in the medium. 2-CP, however, was dechlorinated to phenol when supplied as the sole electron acceptor. While 2,5-DCP was dechlorinated to phenol by the OM enrichment culture; no dechlorination of 2,3-DCP or 2,6-DCP was observed. *Ortho*-dechlorination of 2,4-DCP to 4-CP was supported by this culture, but no TCPs or PCP were utilized. Nitrate and fumarate were not utilized as electron acceptors and did not support growth as measured by VFA depletion.

The electron donors that supported the dechlorination of 2- and 3-CP in the OM enrichment culture are listed in Table 6.1. Although acetate initially

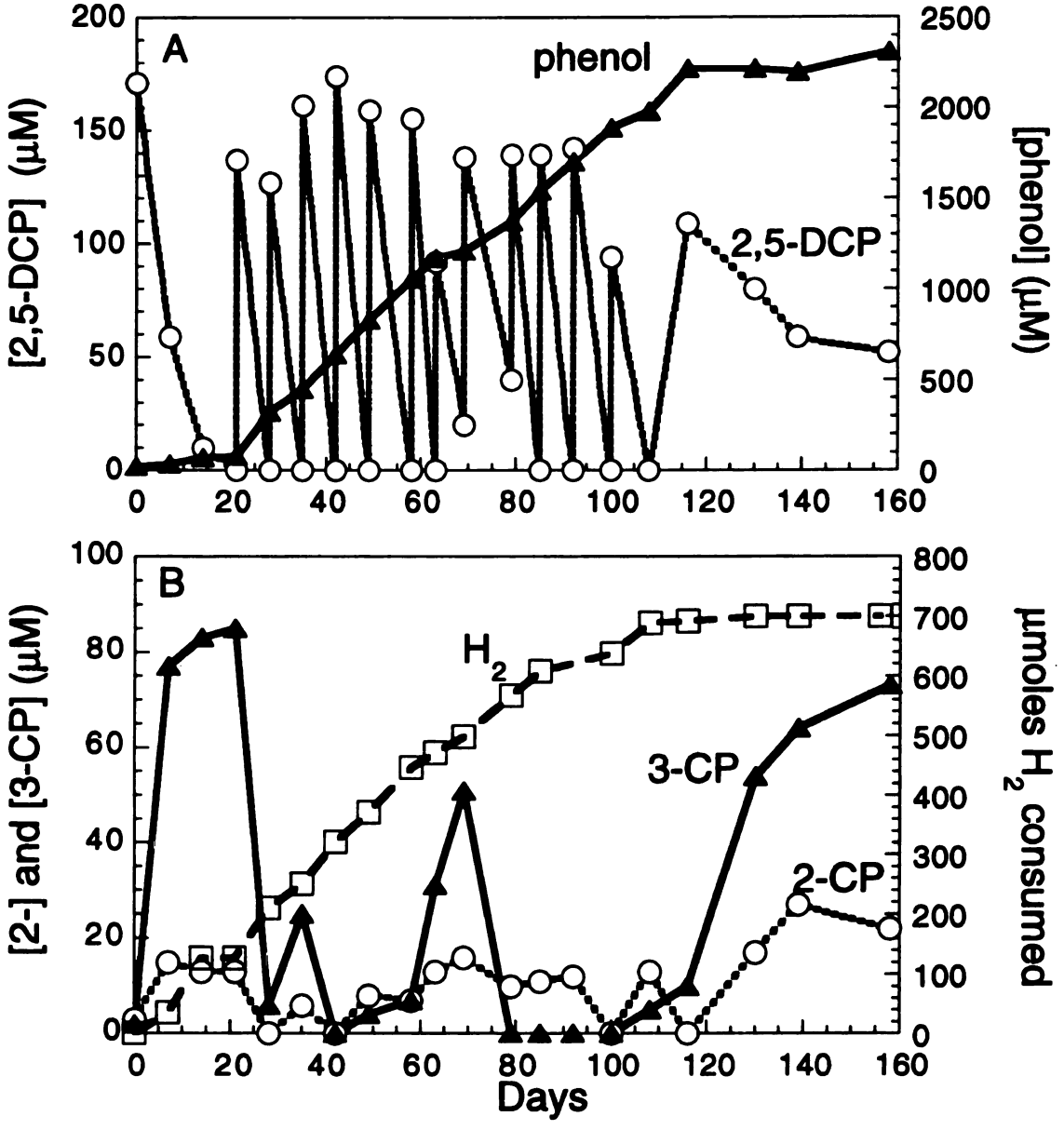

Figure 6.2. Dechlorination of 2,5-DCP by the OM enrichment culture. (A) Shows the dechlorination of 2,5-DCP and the stoichiometric accumulation of phenol. (B) shows the appearance of 2- and 3-CP as intermediates of 2,5-DCP dechlorination and the cumulative H_2 uptake in the OM culture.

Table 6.1. Electron acceptors and donors utilized by OM enrichment culture. A (+) indicates that both the electron donor and acceptor were depleted. Electron donors were tested using 2-CP plus 3-CP as electron acceptors.

Compound tested	Use
Electron acceptors	
2-CP	+
3-CP	-
2- & 3-CP	+
4-CP	-
2-Fluorophenol	-
2-Bromophenol	-
2-Iodophenol	-
2,3-DCP	-
2,4-DCP--> p-CP	+
2,5-DCP--> phenol	+
2,6-DCP	-
2,3,5-TCP	-
2,4,5-TCP	-
PCP	-
3-Cl-4-hydroxybenzoate	-
3-Cl-anisaldehyde[a]	-
Fumarate	-
Nitrate	-
Electron donors	
Acetate	-
H_2	+
Formate ----> H_2[b]	+
Succinate	-
Propionate	-
Butyrate	-
Crotonate	-
Pyruvate[c]	+/-
Lactate	+

[a] 3-Cl-anisaldehyde is a natural product produced by white-rot fungi.

[b] Formate was stoichiometrically converted to H_2 before dechlorination.

[c] Pyruvate was depleted prior to any dechlorination and supported growth in the absence of chlorophenols.

supported dechlorination, after months of incubation in the original enrichment cultures, the enrichment transfers showed no activity with acetate as an electron donor (data not shown). Formate was as good an electron donor as H_2, but measurements of the headspace suggested that formate was converted to H_2 before the start of dechlorination activity. Pyruvate and lactate also supported dechlorination, however, H_2 was measured in the headspace of both these cultures and may have been responsible for the dechlorination activity.

H_2 from formate oxidation supports dechlorination. Since H_2 was observed in formate-fed OM enrichment cultures, the ability to oxidize formate was tested in OM cultures that had been serially transferred eight times with H_2 as the electron donor. This would determine if H_2 production from formate was a stable feature of the OM enrichment culture. Upon feeding formate to the serially-transferred cultures, extensive production of H_2 was seen followed by dechlorination of 2,5-DCP after a considerable lag time (Figure 6.3). Stoichiometric production of hydrogen was observed from formate (data not shown). Rates of dechlorination in formate-fed OM enrichment cultures were, however, slower than H_2-fed cultures, despite the presence of substantial H_2 in the former.

Evidence for halorespiration. As an indicator of whether halorespiration occurs in the OM enrichment culture, the fraction of electrons from H_2 used for reductive dechlorination, **fe**, was determined (Figure 6.4). The **fe** of 0.61 was observed in six replicate cultures. From Figure 6.4 it is apparent that dechlorination did not start in the OM enrichment cultures until 102 µmoles (+/- 5) of H_2 had been consumed. In autoclaved controls no loss of H_2 was observed, indicating that H_2 consumption that occurred prior to dechlorination was biologically mediated.

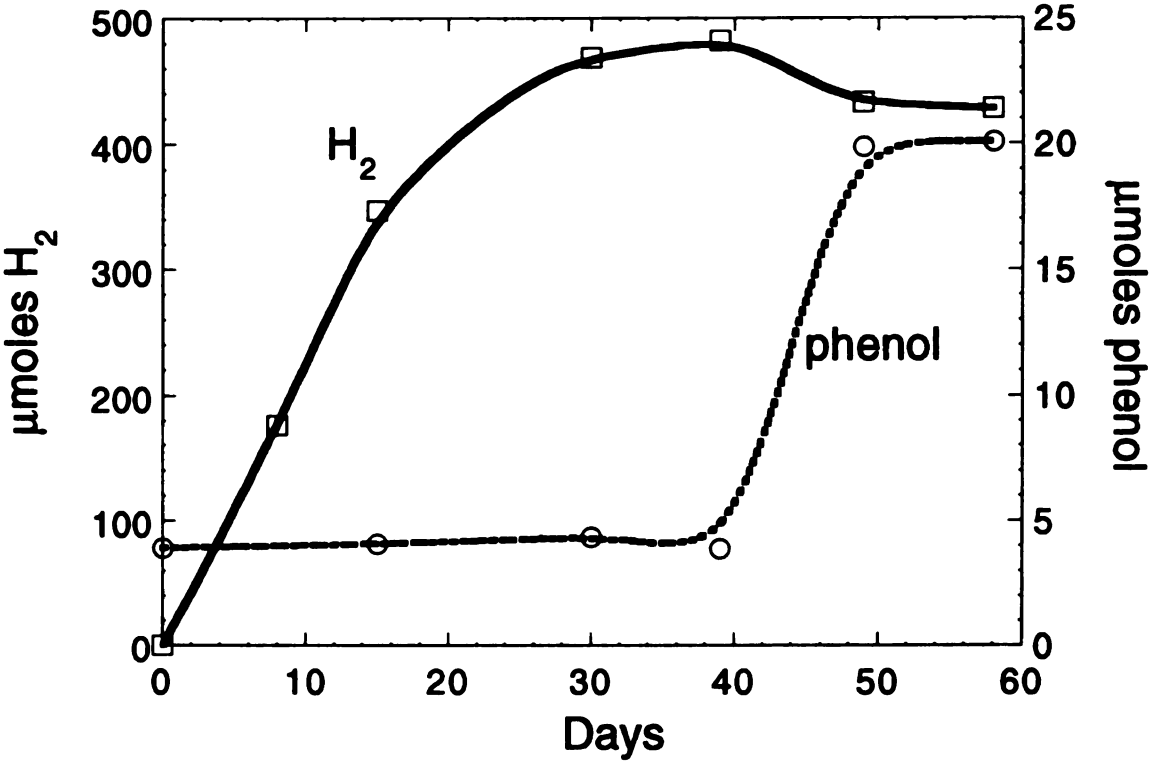

Figure 6.3. Production of H_2 from formate-fed OM enrichment cultures and the dechlorination of of 2,5-DCP as indicated by the appearance of phenol.

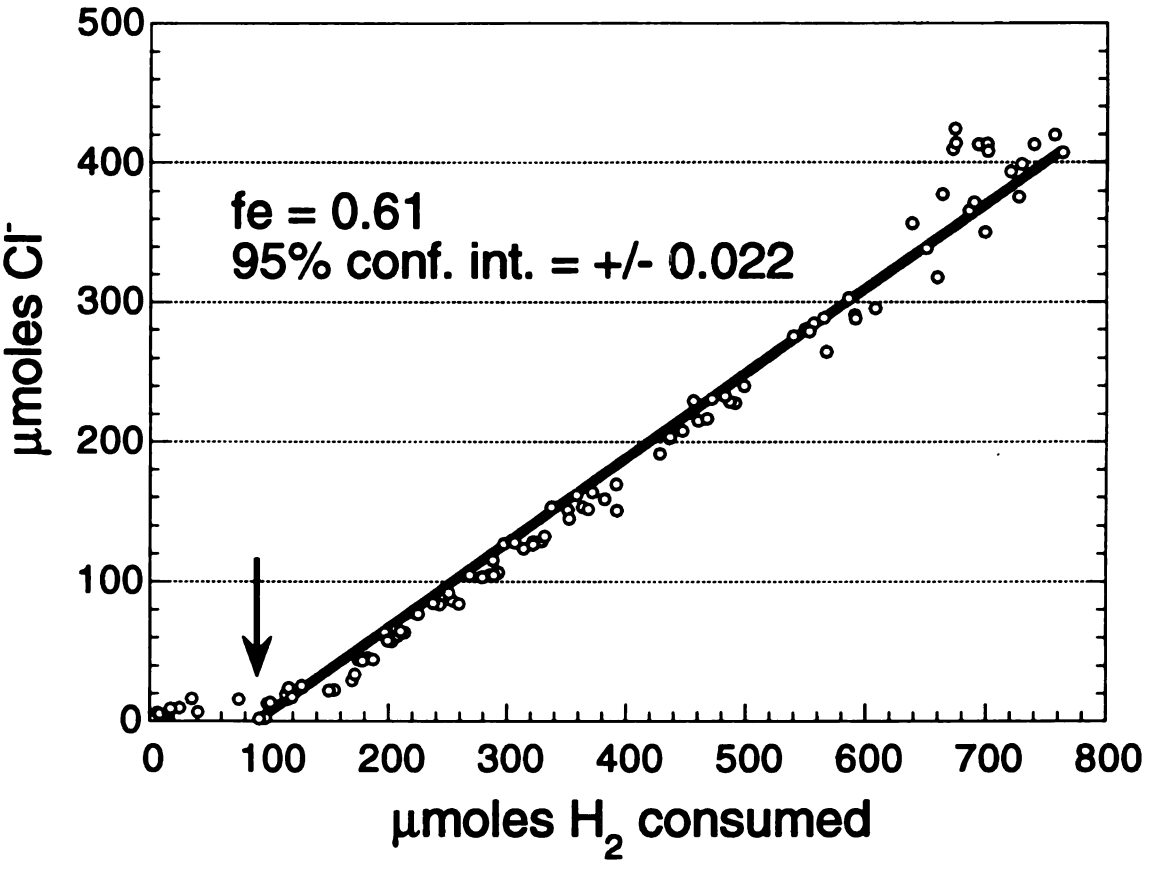

Figure 6.4. The fraction of electrons (**fe**) used for dechlorination in the OM enrichment culture. The **fe** is equivalent to the slope of the regression line of the plotted data. The 95% confidence interval was calculated for six replicate cultures. Dechlorination activity started after an average of 102 µmoles of H_2 had been consumed (arrow).

Additional evidence of halorespiration was provided by the determination of the H_2 threshold concentration. Cultures were starved for H_2 while maintaining the presence of 2,5-DCP as an electron acceptor. The H_2 concentration was monitored until it was below the detection limit of the instrument, <0.5 ppm (Figure 6.5). Rates of H_2 uptake in the OM enrichment cultures were slow, since the cultures were not shaken. Therefore 90 days were required for the culture to approach a final H_2 threshold concentration.

Discussion

Reductive dechlorination of chlorophenols often has exhibited a preferential order for the removal of specific positions from the aromatic ring, with *ortho* > *para* > *meta* (Cozza and Woods 1992; Juteau et al. 1995). The OM enrichment culture, in contrast to these previous studies, is shown to simultaneously remove *ortho*- and *meta*- chlorines from monochlorophenols and 2,5-DCP. H_2 serves as the sole electron donor for this culture. A schematic of the dechlorination pathway exhibited by the OM enrichment culture is shown in Figure 6.6. In addition to the dechlorination from both positions occurring concurrently, two other features of the OM enrichment culture are quite interesting. The culture exhibits no dechlorination activity with 3-CP alone, therefore *meta*-dechlorination activity was induced by the presence of 2-CP. Second, 2,5-DCP, a substrate with chlorines located *para* to each other, is completely dechlorinated to phenol while the *ortho* substituted dichlorophenols 2,3-DCP and 2,6-DCP are not dechlorinated at all. This suggests substrate specificity by the microbial population responsible for the reductive dechlorination of 2,5-DCP. In contrast to the chlorophenol halorespiring cultures, *Anaeromyxo dehalogenans* (2CP strains) and *Desulfitobacterium halorespiricans* strain Co23, *ortho*-chlorines are not

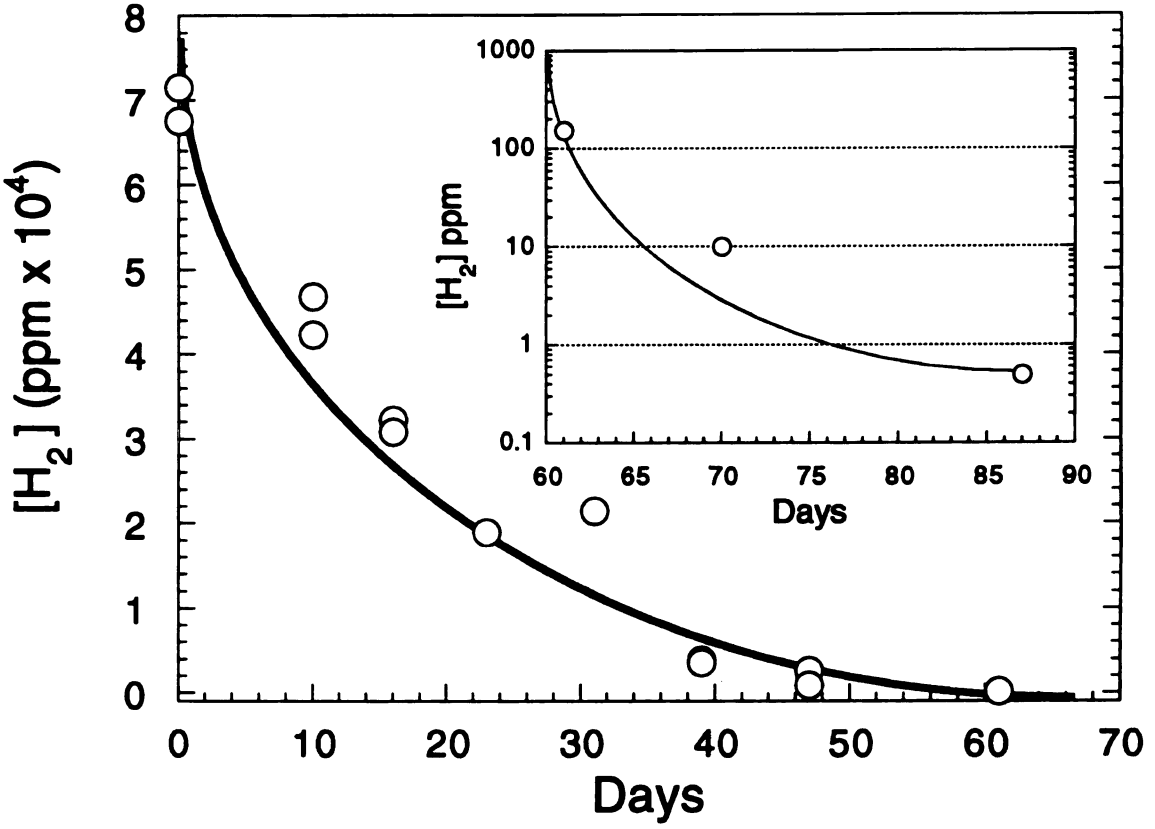

Figure 6.5. Hydrogen uptake and threshold concentrations by the OM enrichment culture. Inset has log scale to show the lower final H_2 threshold concentration.

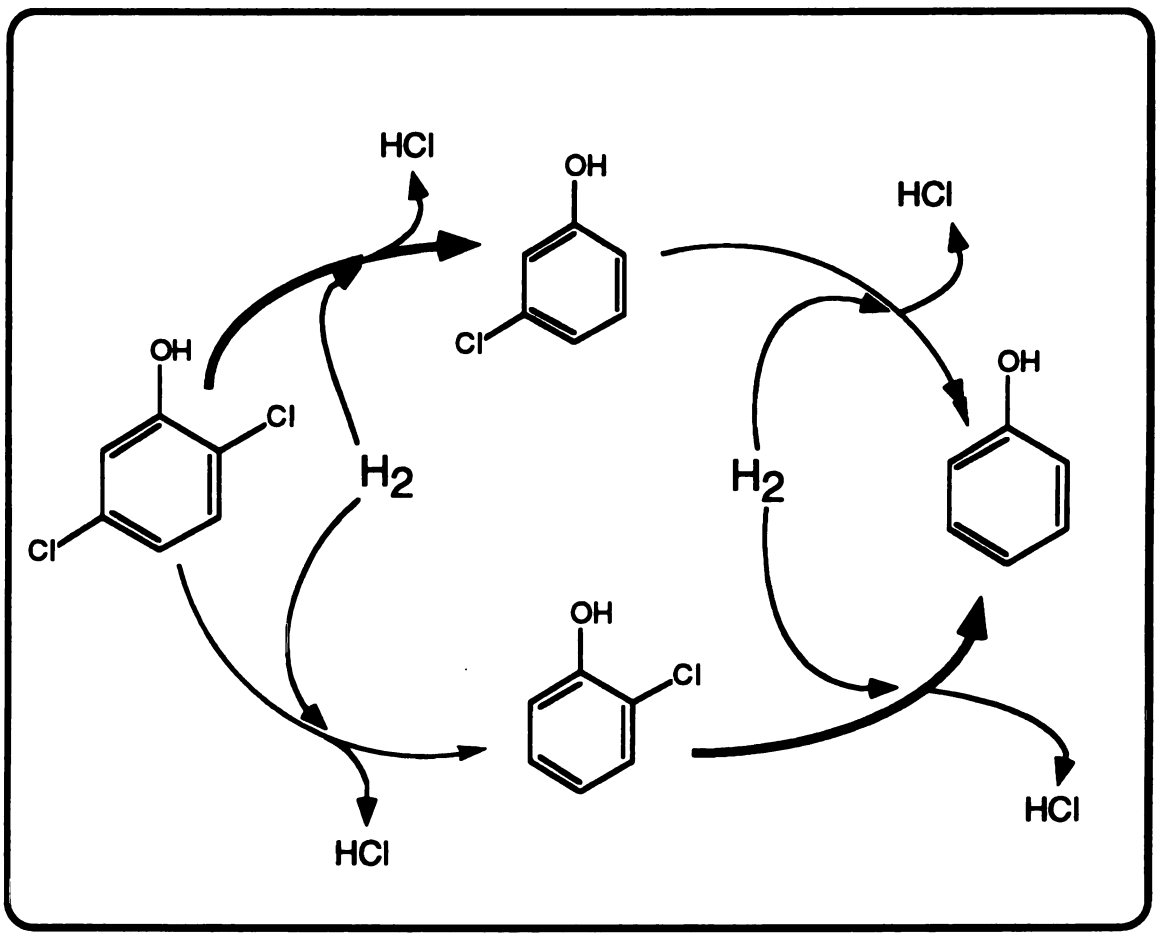

Figure 6.6. Schematic representation of dechlorination pathways of OM enrichment culture. Heavier arrows represent the slightly favored reactions, thus ortho-dechlorination occurs slightly faster than meta-dechlorination.

removed from dichlorophenols with more than two adjacent (chlorine or hydroxyl) substituents (e.g. 2,3-DCP and 2,6-DCP) (Chapters 3 and 4). Since the OM enrichment culture removes *meta*-chlorines as well, it is possible that 2,5-DCP is the natural substrate for dechlorination by the responsible microorganism. 2,5-DCP was identified as a natural compound occurring in the salivary juices of certain grasshoppers (Eisner et al. 1971), so it is possible that sufficient selection pressure has been presence to drive the evolution of enzymes specific for the anaerobic dechlorination of 2,5-DCP.

Evidence that halorespiration occurrs in the OM enrichment culture is apparent in the determination of the **fe**, and the measurement of hydrogen uptake correlated to the presence of 2,5-DCP. The **fe** accounts for the portion of electrons from the electron donor used for the reduction of the electron acceptor (Criddle et al. 1991). A value of 0.61 is calculated for the OM enrichment culture when grown with H_2 and 2,5-DCP, which is similar to the 0.64 and 0.67 observed for *A. dehalogenans* strain 2CP-C and *D. halorespiricans* strain Co23 respectively (Chapters 3 and 4). The **fe** and corresponding **fs**, the fraction of electrons from the electron donor used for biomass, are correlated to the overall energy available from the metabolism of the electron donor and acceptor (Criddle et al. 1991). The **fe** and **fs** values observed from the OM enrichment culture and the other halorespiring isolates are in the range of predicted values for the $\Delta G^{\circ\prime}$ from the overall reaction (McCarty 1975). For example the $\Delta G^{\circ\prime}$ from H_2 oxidation and 2-CP dechlorination to phenol is -236.9 kJ/mol H_2 (Thauer et al. 1977; Dolfing and Harrison 1992). Oxygen reduction, nitrate reduction to nitrite and sulfate reduction to sulfide have $\Delta G^{\circ\prime}$s of -318 kJ/mol H_2, -243 kJ/mol H_2 and -118 kJ/mol H_2 respectively, when coupled to H_2 oxidation. It is apparent that the $\Delta G^{\circ\prime}$ associated with reductive dechlorination of chlorophenolic compounds is

less than O_2, significantly higher than SO_4^{-2}, and very close to nitrate reduction to nitrite. As theoretical **fs** values are based on $\Delta G^{\circ\prime}$ calculations, the corresponding **fe** values for different electron acceptors increase in value as follows: $O_2 < NO_3^- <$ 2-CP $< SO_4^{-2} < CO_2$ (Criddle et al. 1991). McCarty (1971) has shown that these theoretical values for **fe** and **fs** are in general agreement with observations made of different cultures. Therefore it can be inferred from the **fe** data for the OM enrichment culture that the energy available from reductive dechlorination is being coupled to growth.

Additional evidence for halorespiration in the OM enrichment culture is provided in measurements of the apparent threshold concentration for H_2. The hydrogen threshold concentration reflects the minimum amount of energy that an organism requires to grow (Lovley 1985; Lovley and Goodwin 1988). Hydrogen threshold concentrations are therefore correlated with the energy available from the redox couples mediating their growth. In the case of the OM enrichment culture this is H_2 oxidation and the reductive dechlorination of 2,5-DCP reduction to phenol. The threshold concentration of < 0.5 ppm H_2 measured is comparable to that observed for fumarate reduction and slightly greater than found for nitrate respiration (Cord-Ruwisch et al. 1988). However, it is much less than observed for sulfidogens, methanogens and acetogens. The pure cultures *Anaeromyxo dehalogenans* (2CP strains) and *Desulfitobacterium halorespiricans* strain Co23 also had threshold concentrations of H_2 less than 1 ppm (Chapters 3 and 4). DeWeerd et al (1991) measured the comparable threshold concentration of 0.7 ppm for hydrogen with the halorespirer *Desulfomonile tiedjei* strain DCB-1 when grown on H_2 and 3-chlorobenzoate. This indicates that hydrogen threshold concentrations may be reliable indicators of halorespiratory metabolism in pure and mixed cultures where a halogenated substrate is the only electron acceptor provided other

than CO_2. Hydrogen threshold concentrations have been shown to be more correlated to the redox potential of the terminal electron acceptor rather than individual microbial species (Cord-Ruwisch et al. 1988). Ninety days were required by the OM enrichment culture to reach the H_2 threshold concentration. It would have been easy to misinterpret the final H_2 concentration in the OM enrichment culture due to the slow H_2 consumption rate. Thus, it is important to verify that a steady-state H_2 threshold concentration has been achieved.

One interesting feature of the H_2 consumption by the OM enrichment culture is that approximately 100 μmoles was consumed prior to the beginning of dechlorination (Figure 6.4). The low solubility of H_2 in the media precludes the liquid phase alone from being a significant sink for H_2, a result which is corroborated by the autoclaved control cultures. Therefore the initial H_2 uptake must be biologically mediated. One possible explanation is that non-dechlorinating microorganisms, that provide essential co-factors to the dechlorinating population, grow initially and consume most of the H_2. Since the non-methanogenic OM enrichment culture was fed only $H_2 + CO_2$, the possible types of non-dechlorinating coculture organisms is limited to one, a homoacetogen. Acetate addition facilitated the onset of dechlorination when added to the media, however, there was no less H_2 consumed in these cultures prior to dechlorination (data not shown). Other factors produced by acetogens might also be important to the growth of the dechlorinating culture. Also since $H_2 + CO_2$ conversion to formate is energetically exergonic, it is theoretically possible that a microorganism could grow on these substrates in a non-acetogenic process (Chapter 7). Such an organism might also provide essential growth co-factors to the dechlorinating microorganism. It is interesting that the OM enrichment culture exhibits the reverse reaction when formate was provided as an electron donor, conversion to $H_2 + CO_2$, which is also exergonic

under appropriate conditions. Formate was stoichiometrically converted to H_2 + CO_2 by the OM enrichment culture prior to the onset of dechlorination. It was shown that the formate oxidation to CO_2 with the production of H_2 was mediated by the novel sulfate reducer FOX1, which was not able to dechlorinate 2,5-DCP (Chapter 7). These results confirm that the OM enrichment culture has at least two microbial populations: FOX1, perhaps responsible for initial H_2 consumption, and the H_2-consuming dechlorinating population. H_2 may be the only substrate that supports dechlorination in the OM enrichment, since it was observed in the headspace of cultures amended with each of the organic substrates tested as electron donors. This would be similar to *Halobacter restrictus* which only uses H_2 as an electron donor and PCE as an electron acceptor (Holliger et al. 1993).

The prospects of isolating the population responsible for dechlorinating 2,5-DCP in the OM enrichment culture are good. The evidence that halorespiration is occurring suggests that this mechanism could be utilized for selective advantage in an enrichment. The best strategy may be to use a slightly more complex medium (e.g. add yeast extract or rumen fluid) with excess acetate. Any additional cofactors required by the dechlorinating population would then be provided in the medium and the excess acetate would provide a carbon source and possibly inhibit acetogenesis. The unique ability of the OM enrichment culture to mediate simultaneous *ortho*- and *meta*-dechlorination make it a very attractive candidate for isolation and further study.

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Chapter VII

Anaerobic formate oxidation supports growth in H_2 producing microbial culture

All of the work described was done by Robert A. Sanford with the exception of the 16S rRNA sequencing and phylogenetic analysis performed by John W. Urbance. This chapter is formatted as a manuscript for submission to the journal Science.

Abstract

Strain FOX1, a newly isolated microorganism obtains energy for growth by anaerobic oxidation of formate to equimolar amounts of hydrogen and CO_2. Such a reaction was not expected because the free-energy is so low, but exponential growth of FOX1 continued to a $\Delta G'$ of -5 to -10 kJ, providing evidence for a new lower limit of free-energy that supports growth. Furthermore, FOX1 catalyzed the reaction to thermodynamic equilibrium. H_2 was produced at rates of 7300 ml $h^{-1}g^{-1}$ protein and to partial pressures in excess of 100 kPa, suggesting that this bacterium may have economic value as a H_2 source. Analysis of the 16S ribosomal RNA places FOX1 within the family *Desulfovibrionaceae*, in the δ-subdivision of the Proteobacteria. Our work also suggests that the role of formate in anaerobic ecosystems should be reevaluated, particularly its assumed equivalency to H_2.

Hydrogen, a key intermediate in anaerobic ecosystems (1), has been described as the fuel of the future (2) . It is an important feedstock in industrial processes, as well as a source of clean energy (2). Although currently a byproduct from petroleum refining, impurities from this source of H_2 can prevent its use in some processes, like fuel cells. Hydrogen is also produced from the steam reformation of natural gas or methanol, which although a clean process, requires high temperatures and therefore considerable energy input. Biologically generated hydrogen may provide an environmentally sound, energetically efficient, small scale production method that could be an attractive renewable alternative to existing technologies. This is particularly true for H_2 generated from industrial waste treatment. In the past biologically generated hydrogen was not economical in part because of slow production rates and its susceptibility to by-product inhibition (3). In addition light is required in many of

these biological processes, which may be impractical in some circumstances (4). Other technologies being investigated are H_2 from cyanobacterial nitrogenase and the conversion of CO to H_2 and CO_2 (4). Although they show promise these developing technologies either do not produce hydrogen at very high rates or are limited by the solubility of the substrate, e.g. CO. Recently it was shown that good rates of anaerobic fermentative H_2 generation from simulated carbohydrate wastes can be sustained (5). We have isolated strain FOX1 (for formate oxidation), a bacterium that generates H_2 from formic acid in the dark in a energy-yielding process that sustains growth. Since formate is common in anaerobic fermentations, FOX1 may also augment H_2 production in anaerobic waste treatment processes. Sufficient hydrogen was produced to higher partial pressures by this culture and at sustained rates approaching an order of magnitude higher than generally reported for other biological systems (3, 4), and hence may be considered a new approach for hydrogen production.

Hydrogen is also important in anaerobic food webs that usually terminate in methanogenic or acetogenic processes. The generation of methane or acetate requires the presence of a suitable electron source, usually H_2 or formate. Obligate syntrophic microorganisms rely on methanogens to consume the H_2 they produce. This interspecies H_2 transfer is a fundamental component of anaerobic food webs terminating in methanogenesis (6, 7, 8). Many researchers have proposed that formate transfer would be energetically equivalent and more likely than H_2 transfer mainly due to its greater solubility and higher diffusion coefficient (6, 9). Microbiologists and engineers treated these two electron donors as energetically equivalent, because their interconversion is close to thermodynamic equilibrium under standard conditions (10) (Equation 1).

$$HCOO^- + H_2O \longrightarrow HCO_3^- + H_2 \qquad \Delta G^{\circ\prime} = +1.3 \text{ kJ/reaction} \quad \text{Eq. 1}$$

However, formate, H_2 and CO_2 are not likely to be in equilibrium in the environment. At realistic environmental concentrations of reactants and products, this slightly endergonic reaction becomes exergonic. The formate oxidizing isolate we have discovered confirms this theoretical consideration, since it grows using formate as its sole energy source. To calculate the $\Delta G'$ at 30° C we used the following formula:

$$\Delta G'(kJ) = \Delta G^{\circ\prime} + 2.52\ln([HCO_3^-] \bullet [H_2]) - 2.52*\ln[HCOO^-] \quad \text{Eq. 2}$$

Figure 7.1 shows that at pH 7.0 with formate and HCO_3^- at 0.01 M this reaction is exergonic for all H_2 concentrations under 100 kPa (1 atm). The $\Delta G'$ is -21.9 kJ when the initial H_2 partial pressure is 0.01 kPa. Under these initial conditions it is clearly not energetically favorable to convert H_2 and CO_2 into formate. Hence, interspecies H_2 transfer should be favored over formate transfer, when excess formate is present. This implies that there may be a thermodynamic constraint on interspecies formate transfer, since if an organism produces formate, it is energetically favorable to oxidize it to H_2. In Figure 7.1 the energy available from CO_2 reduction to methane with hydrogen is also shown. The area shaded in the middle corresponds to H_2 concentrations, where formate oxidation would support both a formate oxidizing microorganism (like FOX1) and a methanogen. Since this demonstrates that energy is

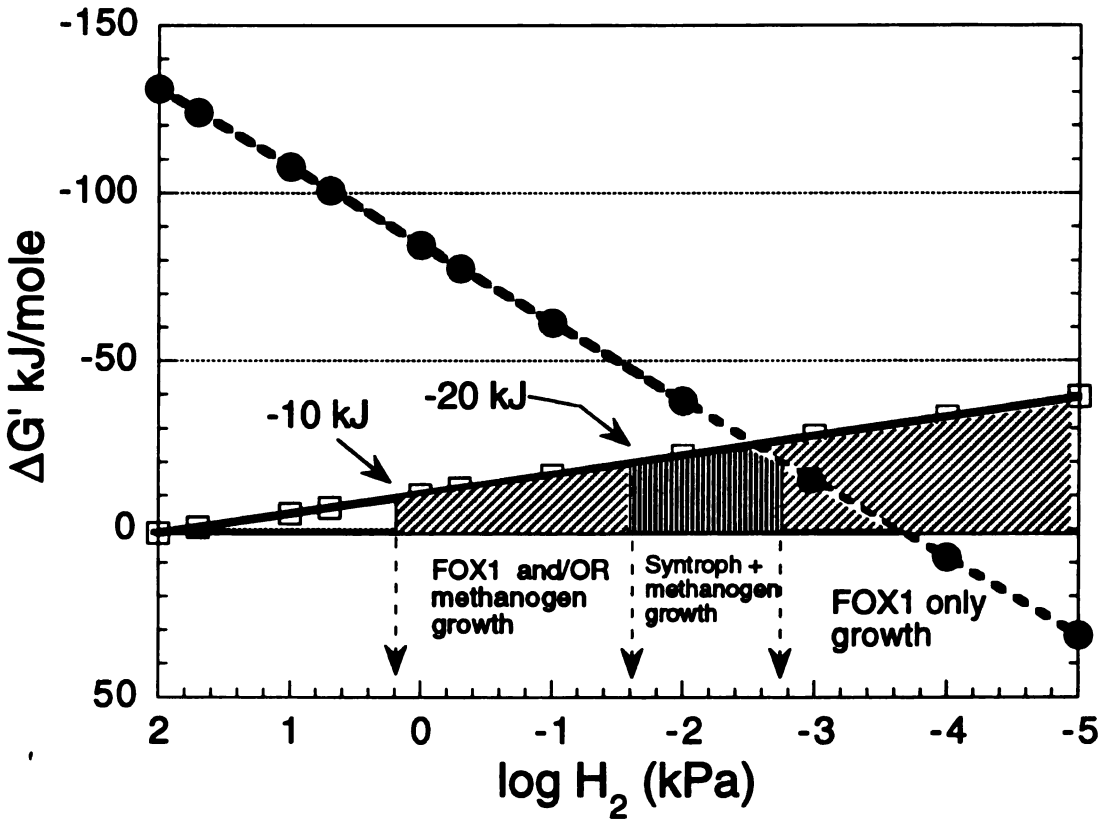


Figure 7.1. The relationship between the H_2 partial pressure and the $\Delta G'$ for formate oxidation (❑) ($HCOO^- + H_2O \rightarrow HCO_3^- + H_2$) and for methanogenesis (●) ($4\,H_2 + HCO_3^- + H^+ \rightarrow 3H_2O + CH_4$). Initial concentrations are: formate = 0.01 M, HCO_3^- = 0.01 M and CH_4 = 10 kPa. The central shaded area shows the H_2 concentration range that would support the growth of a formate oxidizing organism and a methanogen consuming the H_2 in a simple anaerobic community. The adjacent flanking shaded regions indicate the extended H_2 concentration range that corresponded to the $\Delta G'$ associated with the growth of FOX1 alone.

available from formate oxidation, we sought and found a somewhat specialized microorganism that has evolved the capability to couple growth to this process.

FOX1 was obtained from an anoxic enrichment culture fed volatile fatty acids (VFAs), including formate, and that showed reductive dechlorination of chlorophenol. Analysis of the headspace gas of this enrichment showed significant concentrations of H_2 (3.0 kPa), but only trace amounts of methane, which was expected to be the predominant product. Both the dechlorination activity and H_2-producing activity were serially transferred twice in enrichment cultures that were supplemented with a vitamin mixture (450 µg/l) before an effort was made to isolate colonies on anaerobic medium (11). Transfers of this culture have shown that formate alone will support the production of hydrogen, although this activity is stimulated when acetate is added to the medium. This is not unusual for anaerobic cultures, particularly those that have so little energy available. Presumably acetate is used for biosynthesis of cell material. FOX1 was purified from the original enrichment by serial dilution in the formate/acetate medium (12).

To prove that anaerobic formate oxidation supported actual growth of FOX1, direct microscopic counts of acridine orange stained cells were made by taking samples at different time intervals (Figure 7.2 A). Direct counts were done since the total number of cells were not high enough for a turbidity assay. Controls without formate or with H_2 and CO_2 did not show appreciable increases in cell numbers. The doubling time was 30 hours during exponential growth. Autoclaved controls containing cells with formate and acetate exhibited no H_2 evolution(data not shown). Figure 7.2 B shows that as formate was consumed stoichiometric quantities of H_2 were evolved from the same culture. No acetate or other VFAs were produced by this culture. Also shown in Figure 7.2 B is the $\Delta G'$ calculated at different points during the growth of this culture.

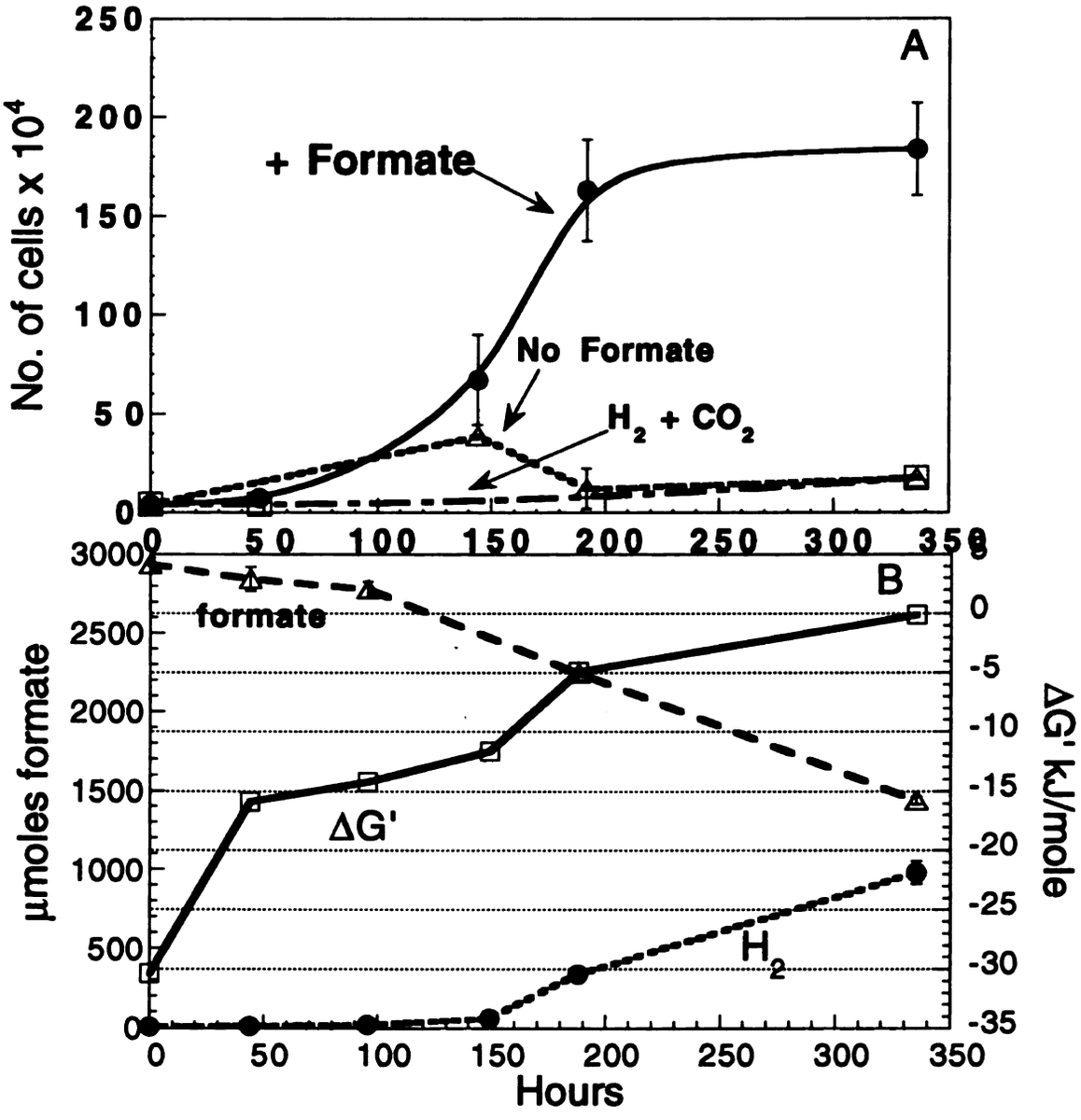


Figure 7.2. **A.** Growth of anaerobic formate oxidizing bacterium FOX1 in broth culture at 30° C as measured by direct microscopic counts of acridine orange stained cells. A known volume of culture was stained and passed through a 0.2 micron filter. Stained cells were then observed under fluorescent illumination and enumerated. **B.** Illustrates the loss of formate from cultures and the subsequent stoichiometric production of H_2. Culture volume was 100 ml with a headspace of 60 ml. Error bars indicate the 95% confidence intervals for triplicate cultures.

Significant growth occurred at ΔG' values between -10 kJ and -5 kJ, at which point exponential increase in cells stopped. This would be a lower growth supporting ΔG than has been observed for any microorganism. A minimum ΔG of -20 kJ had been proposed due to the theoretical quantum of energy required to translocate one proton, which would correspond to one-third ATP (1).

The H_2 evolution rate in batch cultures was 7300 ml $h^{-1}g^{-1}$ protein (0.33 mole $h^{-1}g^{-1}$) and the cell yield was 20.9 μg protein per mmol formate consumed. Microscopic observations of cells in stationary phase showed that FOX1 is pleomorphic after growth on formate (Figure 7.3), while little variation in cell morphology was observed after growth on pyruvate. This may be the due to the stress caused by the limited energy available from formate oxidation.

Preliminary identification and characterization of FOX1 was achieved by phylogenetic analysis using the 16S ribosomal RNA gene sequence (13). Four 16S rDNA clones from the enrichment culture were examined and all had essentially identical sequences. The identical sequences and unambiguous sequence results from the uncloned PCR product suggested that the culture did consist of a single organism. Phylogenetic analysis placed the formate-oxidizing organism within the family *Desulfovibrionaceae*, in the delta-subdivision of the Proteobacteria, as illustrated in Figure 7.4 (14, 15). Its nearest relative was *Desulfovibrio africanus* (88.0% sequence similarity). Preliminary experiments have shown that FOX1 is a sulfate reducer (12) and therefore its physiology is consistent with its phylogeny. It is perhaps not surprising to find this organism related to the delta-subgroup of the Proteobacteria, since this group is particularly noted for its diversity of anaerobic processes.

Since the energy apparently available from anaerobic formate oxidation to hydrogen is minimal (Eq.1), we determined whether thermodynamic

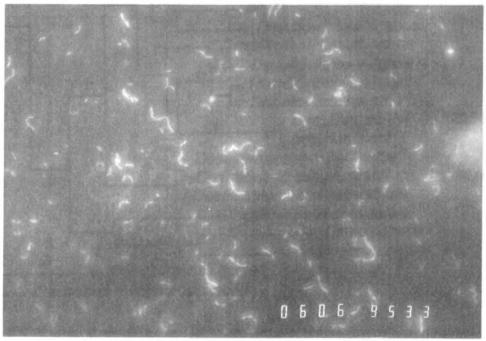
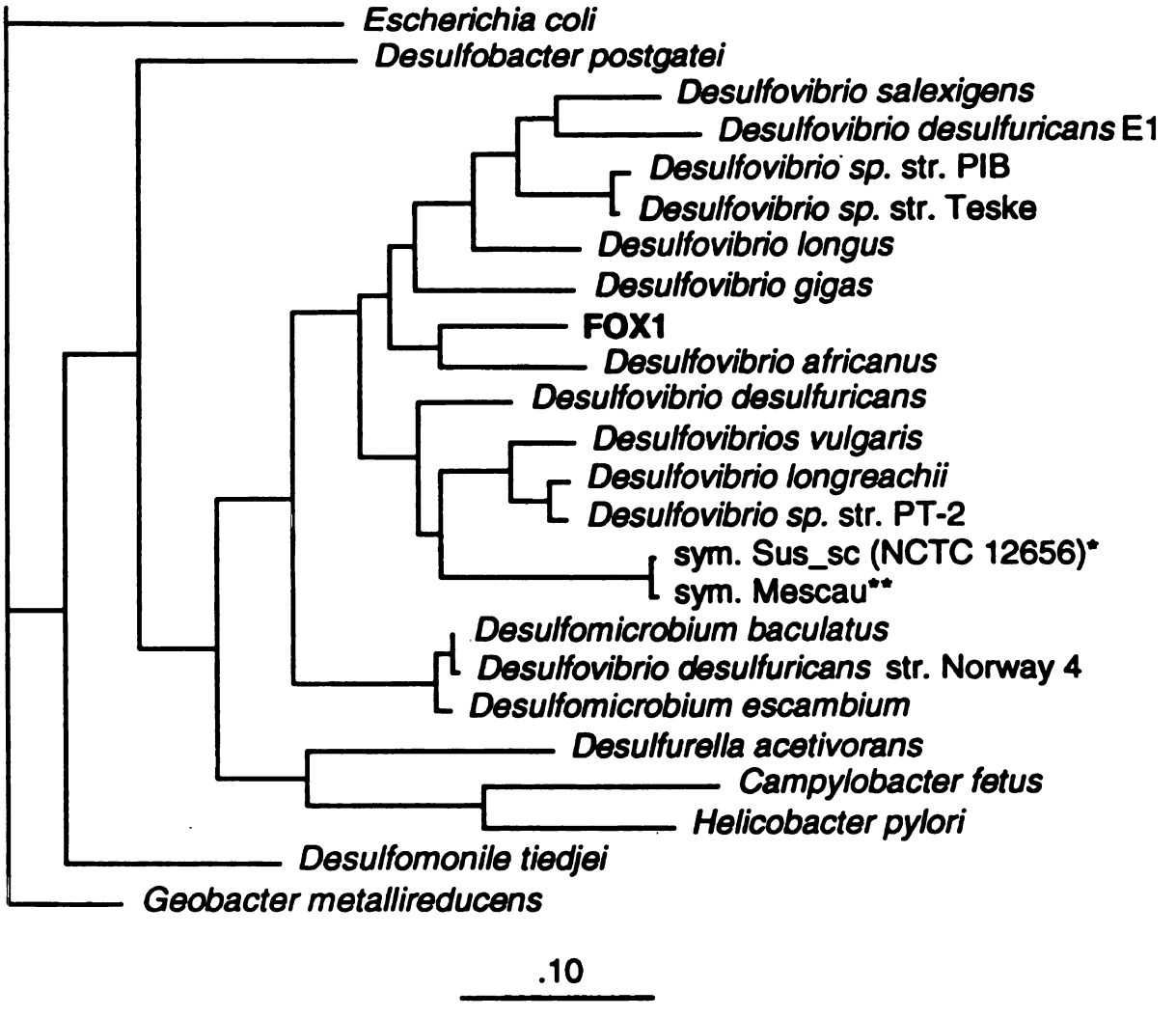


Figure 7.3. Fluorescent microscopic image of acridine orange stained FOX1 bacterium after growth on formate. The average size of cells in the culture is 2 μm wide and 3 to 10 μm long.



* Intracellular ileal symbiont of *Suis scrofus*

** Intracellular ileal symbiont of *Mesocricetus auratus*

Figure 7.4. 16S rRNA phylogeny of FOX1 and related organisms. Near complete (ca. 1500 nucleotide positions) 16S rRNA genes were aligned using the primary and secondary structure with sequences of the nearest relatives obtained from the Ribosome Database Project (RDP). Phylogenetic relationships were inferred by maximum likelihood analysis (15).

equilibrium was reached or whether there was a H_2 threshold concentration beyond which no further formate oxidation would occur. A threshold may be expected in some systems since energy may be required to initiate the process, such as transport of formate. If this were the case the free-energy ($\Delta G'$) would be negative for the system favoring further formate oxidation even after H_2 evolution ceases. To determine this, cultures of the H_2 producer were initiated with different initial formate concentrations: 15 mM, 30 mM and 60 mM. Results showed that as the initial formate concentration increased the final H_2 concentration increased to as high as 66 kPa with the 60 mM formate culture (Figure 7.5 A). In fact H_2 concentrations of greater than 100 kPa were obtained with similar cultures that had been refed formic acid. Regardless of the initial formate concentration the calculated $\Delta G'$ at the end of incubation was approximately 0 kJ/mole, indicating that thermodynamic equilibrium had been obtained (Figure 7.5 B). Most of the available free-energy was used during initial formate oxidation when no H_2 was present. For example, when equation 2 is calculated after the reaction has partially proceeded, with formate equal to 0.009 M, HCO_3^- at 0.011 M and H_2 equal to 3.7 kPa, the $\Delta G'$ increases from -21.9 kJ to -6.5 kJ (assuming a 100 ml culture with a 60 ml headspace). At equilibrium under the same conditions, the H_2 partial pressure reaches approximately 18.5 kPa, illustrating that most of the H_2 evolution exhibited by this organism does not yield much energy. The ability to easily determine the free-energy under initial conditions and at equilibrium is perhaps unique for a biological process that yields energy for growth and hence may be a good model for studying the coupling of free-energy to growth.

Our observations demonstrate the feasibility of formate serving as an energy source for anaerobic growth, with stoichiometric production of hydrogen. Since FOX1 produces H_2 at sustained rates up to ten times those of other

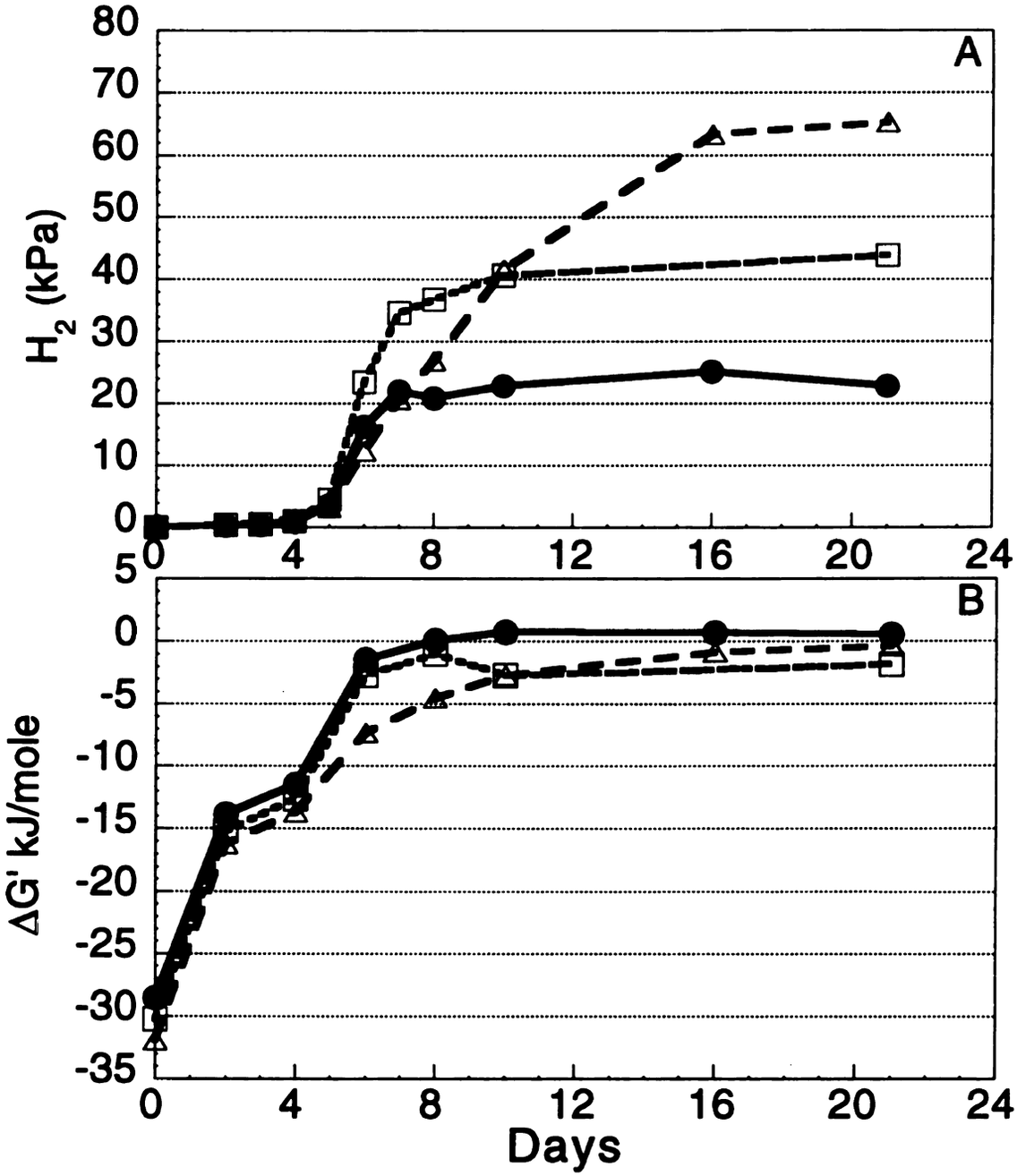


Figure 7.5. Hydrogen production and free energy change in duplicate cultures started at three formate concentrations: 15 mM(●), 30 mM(❑) and 60 mM(Δ). **A.** Graphically represented is the average partial pressure for H_2 in duplicate cultures. Hydrogen concentration reached a steady state in all cultures after 20 days. **B.** Free energy ΔG' is shown for the same cultures with different initial formate concentrations. The ΔG' was calculated by measuring the H_2 partial pressure and determining by difference the formate and bicarbonate concentrations. Formate was measured initially and at the final data point to verify the free-energy calculations. All three cultures converge at thermodynamic equilibrium at 20 days.

biological systems (4), it may provide a more attractive method for industrial H_2 production. Although many microorganisms, including *E. coli*, can convert formate into H_2, this reaction has not been described as energetically beneficial and independent of other energy yielding substrates (16, 17). The discovery of this culture suggests that some of the previously described H_2-evolving, formate-oxidizers should be reevaluated for the potential energetic benefit of this process, particularly other sulfate-reducing δ-Proteobacteria. The *Desulfovibrio* are known to contain a variety of hydrogenases and are known to possess the low-potential electron carriers, like ferrodoxins, that would be required for the energetically favorable generation of H_2 (18), which may then be coupled to cell growth. The positioning of hydrogenases across the membrane is one mechanism that may explain growth of FOX1 at a $\Delta G'$ greater than -10 kJ, since H_2 diffusion out of a cell could serve as a type of proton translocation with little energetic cost.

The role of formate in anaerobic food webs, particularly its possible role in interspecies electron transfer, should be reevaluated. For example in a methanogenic ecosystem with a significant formate flux, the formate oxidation to methane and CO_2 may actually support the growth of two microbial populations: the FOX1-type H_2-producer and the methanogen (Figure 7.1). The only requirement for this type of consortium to function is to have formate not in equilibrium with H_2 and CO_2. Such an environment was described by McMahon and Chapelle when they found that high concentrations of formic and acetic acid are produced in aquitard sediments and subsequently diffuse into anaerobic sulfidogenic and methanogenic aquifer material (19). Since H_2 concentrations in such anaerobic environments are kept very low by methanogenesis and sulfate reduction, the oxidation of formate to H_2 would be

thermodynamically favorable. Therefore reasonable conditions do exist in the natural environment for the anaerobic oxidation of formate to hydrogen.

References and Notes

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12. Formate grown cultures were transferred eight times with 0.1 % (v/v) per transfer. After this three successive serial dilution series to extinction of H_2 generation were done to purify FOX1. Since so little energy is available from formate oxidation, dilutions of 10^{-6} were sufficient to achieve purity. The purity of FOX1 was verified microscopically by observing cells grown on pyruvate (a common fermentation substrate) and lactate plus sulfate (a common substrate for sulfidogens). Pyruvate was fermented to acetate and succinate while sulfate was reduced to sulfide by FOX1. Observations verified the presence of a single morphotype and both of these rich-media cultures were successfully transferred back to a formate medium which showed H_2 production activity. Also pyruvate grown FOX1 was subjected to DNA extraction and PCR amplification of 16S rDNA. Only one sequence, was observed which was identical to the sequence found for FOX1 grown on formate. Efforts to grow FOX1 on agar medium have been unsuccessful.

13. A replicate of the formate-oxidizing culture was harvested by centrifugation. Cells were lysed by repeated freeze/thaw cycles and the nucleic acids purified by phenol/chloroform extraction and alcohol precipitation. Near complete (ca. 1500 bp) 16S rRNA genes were then PCR amplified using previously described primers. J. Zhou, M. R. Fries, J. Chee-Sanford, J. M. Tiedje, *Int. J. Syst. Bacteriol.* **45**, 500-506 (1995). A fraction of the amplified product was cloned using a commercial kit (TA Cloning Kit. Invitrogen. San Diego, CA.) and four of the resulting clones were then randomly selected and sequenced, along with the uncloned, amplified product.

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Chapter VIII

SUMMARY AND CONCLUSIONS

In the concluding chapter I will summarize how my research has answered my primary research questions and the importance of certain concepts that have emerged from this work. In doing so, the contribution of individual chapters to the synthesis of those concepts will become apparent.

Four research questions were addressed during this study (Chapter 1). The answer to the first question appears to be that denitrifiers do not exist that can dechlorinate and degrade chlorophenols. My results more thoroughly evaluated this trend noted from other research (Chapter 2), although it is very difficult to conclude that this is globally true. It is still a reasonable assertion that some microorganism that is difficult to culture may be capable of both of these activities and therefore an absolute statement cannot be made. At best such organisms are rare or are difficult to enrich.

The second research question was to identify the microbial populations responsible for reductive dechlorination of chlorophenols. Five different dechlorinating activities were identified in enrichment cultures (Chapter 2). Four of these dechlorinating cultures were chosen for further experimentation, which resulted in the isolation of the anaerobic myxobacterium strain 2CP-C (Chapter 3) and *Desulfitobacterium chlororespirans* strain Co23 (Chapter 4). These isolates exhibited the novel capability of halorespiration using chlorophenolic compounds as a terminal electron acceptor. The other two dechlorinating cultures exhibited distinctly different dechlorination patterns, but

did not yield isolates (Chapters 5 and 6). In the case of the meta-dechlorinating methanogenic enrichment culture (Chapter 5), halorespiration did not appear to occur since most of the available electrons from the added electron donor pool were converted to methane. Since the energy from reductive dechlorination is more favorable than methanogenesis, it is not likely that this process was coupled to growth in these enrichment cultures. Of the five dechlorinating types of cultures identified, four were enriched from the same type of microcosm, one containing a Michigan compost soil. This suggests that considerable diversity of microorganisms capable of reductive dehalogenation exists in non-polluted and not strictly anaerobic environments.

The third research question was to determine the energetic value of reductive dechlorination for the microbial populations in the enrichment cultures. Cultures of strains 2CP-C and Co23 could be studied by monitoring growth coupled to the reductive dechlorination of a chlorophenolic compound, however this is more difficult to assess in enrichment cultures. I found that the measurement of the H_2 threshold concentration and the fraction of electrons from the electron donor used for reductive dechlorination (**fe**), were good indicators that growth was coupled to this process. For strains Co23, 2CP-C and the OM enrichment culture the **fe** was between 0.6 and 0.7 (Chapters 3,4 and 6), while the **fe** of the methanogenic enrichment culture was only 0.01, indicating a minor flux of electrons to reductive dechlorination (Chapter 5). When compared to other electron accepting processes the **fe** for the reductively dechlorinating pure cultures was between that seen for denitrification and sulfate reduction. This is in the predicted range if halorespiration is occurring. The H_2 threshold concentration has been proposed as an indicator of the microbial redox couple controlling particular environmental conditions. If reductive dechlorination is supporting a respiratory process, a H_2 threshold

range is predictable based on comparisons to other electron acceptors. Thus H_2 thresholds would increase for different electron acceptors in this order: O_2 < NO_3^- < chlorinated organic compounds < SO_4^{-2} < CO_2 (methanogenesis). Results of H_2 measurements from strains 2CP-C, Co23 and the OM enrichment culture all showed H_2 concentrations less than 1 ppm (Chapters 3, 4 and 6). This is in agreement with the predicted range of concentrations for halorespiration. In addition, the demonstration of H_2 serving as an electron donor for reductive dechlorination is also indicative of a respiratory process. To more thoroughly evaluate this principle, it would be useful to compare the **fe** and H_2 threshold concentrations of other reductive dechlorinating cultures with the cultures that I studied. This would more clearly establish these measurements as good indicators of halorespiration.

My evidence suggests that the cultures that I have characterized have an apparent preference for relatively low electron acceptor concentrations. In Chapter 3 I introduce the term microrespirotrophy to describe this behavior. It is possible that all the reductive dechlorinating populations are microrespirotrophs, like strain Co23 or the dechlorinators present in the methanogenic and OM enrichment cultures (Chapters 4, 5 and 6). None of the anaerobic dechlorinating cultures tolerated chlorophenol concentrations above 200 μM very well. Even other electron acceptors could not be supplied in concentrations much above 1 mM. The main point I wish to stress is that the use of excessive electron acceptor concentrations in enrichments or isolation protocols may reduce the number of apparently culturable microorganisms. It is quite likely that most microorganisms in nature are microrespirotrophs, simply because electron acceptor concentrations are rarely excessive. This suggests that strategies for culturing new microorganisms may be more successful if low concentrations of electron acceptor are used. Even those organisms that can

use higher concentrations will still likely be able to grow at the lower concentrations, so the benefit would be to maximize the recovery of culturable organisms.

The final research question was to assess the influence of electron donors on anaerobic food-webs terminating with reductive dechlorination and particularly to investigate the relationship between formate and H_2 in anaerobic ecosystems. Specificity for different electron donors supporting reductive dechlorination was evident among the different dechlorinating cultures (Chapters 3,4,5 and 6). This suggests that the diversity of dechlorinating microorganisms is not only dependent on the electron acceptor specificity but also on the ability to use different electron donors. For example, strains 2CP-3 and Co23 both use 2,6-DCP as an electron acceptor and were isolated from the same environment. As unique electron donors, strain 2CP-3 can use acetate and strain Co23 can use butyrate, thus it is theoretically possible to maintain a co-culture of these organisms on 2,6-DCP and butyrate, since Co23 oxidizes butyrate to acetate and can only remove one chlorine. In the case of the meta-dechlorinating methanogenic enrichment culture different electron donors supported different efficiencies of reductive dechlorination with formate serving as the most effective substrate for reductive dechlorination activity (Chapter 5). Formate was also the best electron donor for the OM-enrichment culture, but was converted to H_2 and CO_2 prior to reductive dechlorination, suggesting that H_2 was the true electron donor (Chapter 6). This led to the isolation of strain FOX1, the microorganism capable of growing at the expense of formate oxidation and H_2 generation (Chapter 7). Strain FOX1 was shown to grow at $\Delta G'$ in the range of -10 kJ to -5 kJ per mole of formate, higher than the reported minimum $\Delta G'$ thought necessary for growth. Ecologically, this novel ability in strain FOX1 implies that formate and H_2 are not equivalent in the environment

and that some organisms may take advantage of the energy available from their disequilibrium. In the OM-enrichment culture FOX1 was cross-feeding a dechlorinating microorganism, however the cell-yields were always low. Theoretically it should be possible to co-culture FOX1 with a non-formate-utilizing methanogen and demonstrate an increase in cell-yield of FOX1. This would demonstrate the potential importance of this type of activity in the an anaerobic food-web.

Research in the future on this topic should focus on two areas. First the role of halorespiration in anaerobic environments needs to be more completely investigated. Particularly important is the determination of what naturally occurring chlorinated organic compounds serve as electron acceptors. By focusing the research toward natural ecosystems the diversity of microorganisms capable of halorespiration may be more thoroughly determined. I would recommend the second area focus to be on formate oxidation resulting in the generation of H_2. From the preliminary studies with FOX1 it is evident that a viable economic method for generating H_2 may be developed using these types of microorganisms. This is especially true if anaerobic digestion of waste material can be channeled and enriched for formate production leading subsequently to H_2 generation. Studying the mechanisms associated with formate oxidation by FOX1 and similar types of organisms would also facilitate the development of these anaerobic H_2 generating systems.