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Induction characteristics of reductive dehalogenation in the *ortho*-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*

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Abstract

Anaeromyxobacter dehalogenans strain 2CP-C dehalogenates *ortho*-substituted di- and mono-halogenated phenols and couples this activity to growth. Reductive dehalogenation activity has been reported to be inducible, however, this process has not been studied extensively. In this study, the induction of reductive dehalogenation activity by strain 2CP-C is characterized. Constitutive 2-chlorophenol dechlorination activity occurs in non-induced fumarate-grown cells, with rates averaging 0.138 μmol of Cl^{-} h^{-1} mg of protein^{-1}. Once induced, these cultures dechlorinate 2- chlorophenol (2-CP) at rates as high as 116 μmol of Cl^{-1} h^{-1} mg of protein^{-1}. Dechlorination of 2-CP is induced by phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, and 2-bromophenol. Of the substrates tested, 2-bromophenol shows the highest induction potential, yielding double the 2-chlorophenol dechlorination rate when compared to other inducing substrates. No induced dechlorination is observed at concentrations less than 5 μM 2-CP. When fumarate cultures were diluted 100-fold, fumarate reduction rates were reduced roughly according to the dilution factor, while dechlorination rates were similar in fumarate grown cells amended with 2-CP and cells diluted 100-fold prior to the addition of chlorophenol. This indicates that the majority of the fumarate-grown cells in late log phase were not induced when exposed to inducing substrates such as 2-CP. This observation may have ramifications on the success of bioaugmentation using halorespiring bacteria, which traditionally relies on growing cultures using more readily utilized substrates. The rapid dechlorination rate and unique induction pattern also make strain 2CP-C a promising model organism for understanding the regulation of reductive dehalogenation at the enzymatic level.

Introduction

Halogenated aromatic compounds have been used extensively in several industries and have been released into the environment in substantial quantities (Fetzner 1998). Many of these contaminants have accumulated in groundwater and sediments, where anoxic conditions typically persist. Reductive dechlorination is considered an important mechanism for microbial detoxification and biotransformation of these chlorinated contaminants under anaerobic conditions (Mohn & Tiedje 1992). Of particular interest are halorespiring bacteria, capable of coupling the reductive dehalogenation to energy metabolism and hence to microbial growth. As halorespiring reductive dechlorination rates in general are several orders of magnitude higher than co-metabolic reductive dechlorination rates (Fetzner 1998), their impact on anaerobic bioremediation is potentially important.

A number of pure cultures capable of reductive dechlorination of chlorophenols (CPs) have been isolated and characterized. Among these isolates, it has been observed that dechlorination activities are induced when one or a few chlorinated aromatic compounds are present (Madsen & Aamand 1992; Mohn & Kennedy 1992; Bouchard et al. 1994; Cole et al. 1994; Utkin et al. 1995; Löffler et al. 1996; Sanford et al. 1996; Dennie et al. 1998; Sun et al. 2000). Sev-

eral of these anaerobic pure strains have been used for biotechnological applications as a source of inoculum for the clean-up of environmental sites contaminated by chlorophenols or for cultivation and immobilization in bioreactors used to treat waste streams containing chlorophenols (Mikesell & Boyd 1988; Christiansen & Ahring 1996; Miethling & Karlson 1996; El Fantroussi et al. 1997). These processes require a large quantity of active dechlorinating cells before they can be effectively applied to the contaminated sites or introduced to bioreactors. Understanding cell physiology and enzyme induction is important to maximize active biomass. Recent efforts have focused on the purification of reductive dehalogenases and characterization of components of the energy conservation systems in halorespiring bacteria (Ni et al. 1995; Löffler et al. 1996; Christiansen et al. 1998; Miller et al. 1998; Neumann et al. 1998; van de Pas et al. 1999). Other studies have focused on the molecular mechanisms involved in the regulation of reductive dehalogenation (van de Pas et al. 1999; Smidt et al. 2000). Insight into the physiological aspects of the induction of reductive dehalogenation activity, however, is still limited.

In this work, we characterized the induction of reductive dehalogenation of halophenols in a facultative anaerobic bacterium, *Anaeromyxobacter dehalogenans* strain 2CP-C (Sanford et al. 2002). Strain 2CP-C can grow by coupling the oxidation of acetate to the reductive dehalogenation of *ortho*-halophenols. Besides halogenated phenols, oxygen, nitrate, nitrite, and fumarate are also used by strain 2CP-C as electron acceptors (Sanford et al. 2002). We present evidence that although some constitutive reductive dechlorination does occur, the induction of this activity yields rates at least five-fold higher than background dechlorination rates and are the highest reported, to the best of our knowledge, among all chlororespiring organisms. The extent of this dechlorination activity is apparently dependent on the inducing substrate and its concentration.

Materials and methods

Chemicals

The following halogenated compounds were used in this study: 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP), 2,6-dichlorophenol (2,6-DCP), and 2-bromophenol (2-BrP), all of which were obtained from Aldrich, Milwaukee, WI.

Medium Formulation and Preparation

A chloride-free anaerobic mineral salts medium was used for all cultures (Sanford et al. 1996). The medium contained the following (per liter): 0.5 g of $MgSO_4 \cdot 6H_2O$, 0.632 g of NH_4HCO_3, 0.3 g of K_2SO_4, 0.015 g of $CaSO_4 \cdot H_2O$, 0.5 g of KH_2PO_4, $\cdot 6H_2O$, 0.05 mg of resazurin, 1 ml of trace element solution, 1 ml of selenium-tungsten solution, 0.048 g of $Na_2S \cdot 9H_2O$, 0.035 g of L-cysteine, and 0.084 g of $NaHCO_3$. The trace element solution contained the following (per liter): 1.5 g of $FeCl_2 \cdot 4H_2O$, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 6 mg of H_3BO_3, 36 mg of $Na_2MoO_4 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, and 1.0 mg of $CuCl_2 \cdot 2H_2O$. The selenium-tungsten solution contained 6 mg of Na_2SeO_3 per liter, 8 mg of $Na_2WO_4 \cdot 2H_2O$ per liter, and 0.54 g of NaOH per liter. Sulfide and cysteine were added as reductants to the medium after it had been boiled and cooled to room temperature. The headspace of the medium container was continuously flushed with an oxygen-free nitrogen/CO_2 gas mix, and the pH was adjusted to 7.0 $\pm$ 0.1 by varying the CO_2 composition of the mix. A vitamin solution (1%) (Wolin et al. 1963) and growth substrates were added from sterile anaerobic stock solutions after autoclaving.

Organism and growth conditions

Anaeromyxobacter dehalogenans strain 2CP-C (ATCC BAA-259) was routinely 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 and aluminum seals. Cultures were incubated in the dark at 30 °C under anaerobic conditions (100% N_2 gas headspace). For routine cultivation, acetate (2 mM) was added as electron donor and fumarate (5 mM) was added as the electron acceptor. The culture was maintained by transferring a 1% inoculum to fresh media after fumarate was depleted and growth had stopped. Strict anaerobic techniques were used throughout in experimental manipulations. Sterile syringes and needles, used for substrate addition and sampling, were flushed with N_2 prior to use.

Induction and dechlorination with various CPs

Non-induced cultures of strain 2CP-C, defined as not being exposed to CPs or aromatic compounds for at

least 15 transfers, were grown in mineral salts medium containing 5 mM fumarate and excess acetate (4 mM). During late-log-phase growth (optical density at 600 nm = 0.08 and protein = 26 mg as bovine serum albumin (BSA) L^{-1}), 130 μM of individual halophenols (2-CP, 2,4-DCP, 2,5-DCP, 2,6-DCP, or 2-BrP) were added to these cultures, to evaluate them as inducers of dechlorination activity. Triplicate cultures were used and the initial rate of dehalogenation was measured for each substrate to give a measure of constitutive activity. After 24 hours of incubation at 30 °C and after the inducing substrate was depleted (except for 2,4-DCP), 130 μM 2-CP was added to all the cultures to test the level of induced activity. The rate of 2-CP dechlorination was measured by taking samples every 30–60 minutes over a three hour period after substrate addition and quantifying the loss of substrate and the increase in phenol, the product of dechlorination. At 48 hours after induction, 2-CP (130 μM) was added again and an additional rate determination was done.

Phenol was added directly to the acetate-fumarate growth media to determine if its presence would induce dechlorination prior to the addition of 2-CP. This was done by amending phenol (50 μM) to fresh fumarate media at inoculation instead of the late log phase. After reaching late log phase, 130 μM 2-CP was added and the dechlorination rate was measured and compared to the previously measured constitutive dechlorination rate.

Dechlorination rates were determined using a linear fit to the dechlorination data. To exclude rate increase caused by growth, only data within 3 hours from the addition of CP were used to determine the dechlorination rate. Dechlorination data points during these short periods were typically linear and taken at times sufficiently less than the doubling time of approximately 12 hours (Sanford et al. 2002). Biomass concentration during these experiments was constant as determined by no increase in the optical density (600 nm) and confirmed by protein analysis.

Induction threshhold concentration of 2-CP

The minimum concentration of 2-CP that would show detectable levels of induced dechlorination was tested by varying the concentration of the 2-CP added along with acetate and fumarate to non-induced cultures. The following concentrations were tested: 50 μM, 5 μM, 0.5 μM, 0.05 μM, and a control with no 2-CP. To test the level of induction, 130 μM 2-CP was added when cultures reached late log phase and when the fumarate had been depleted. The dechlorination rates were measured after the addition of 2-CP during late log phase. The dechlorination rates were also measured after two more additions of 130 μM 2-CP at 24 and 48 hours to evaluate any sustained benefit from the initial induced activity. Biomass concentrations during these experiments remained at a constant optical density.

Dechlorination activity and fumarate utilization in dense and dilute cultures

Non-induced dense cultures were grown and prepared as with the other induction experiments. To obtain dilute cultures, dense cultures were diluted 100 fold in fresh media. To both dense and dilute cultures, 130 μM of 2-CP and 4 mM of acetate was added to induce dechlorination activity during late log-phase growth. A second addition of 2-CP was made 24 hours after the initial 2-CP had been depleted. Dechlorination rates were monitored by measuring the depletion of a third addition of 130 μM 2-CP, added 24 hours after the second addition. To test the relative rates of fumarate reduction, fumarate was added to both dense and dilute cultures to final concentrations of 5 mM. Fumarate concentrations in dense and dilute cultures were monitored as described below to determine rates of utilization.

Analytical methods

Chlorinated aromatic compounds were analyzed on a Hewlett Packard 1090 HPLC with a Chemstation analysis software package and a BioRad Hi-Pore reversed phase column. The eluent was phosphoric acid (0.1%) and 50% methanol pumped at 1.0 ml/min. Peaks were quantified at 218 nm on a diode array detector. Samples (1 ml) from the cultures were taken, made basic with 10 μl of 2N NaOH, and filtered through Millipore GSWP 0.20-μm filters prior to HPLC analysis.

Fumarate and other volatile fatty acids were analyzed using a Waters 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 (Sanford et al. 2002). Previously filtered samples were acidified to 0.2 N H_2SO_4 by adding 100 μl of 2 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.

To measure the protein content of the cultures, samples of cell suspension were heated with an equal

volume of 2 N NaOH in a water bath at 90 °C for 15 minutes. The protein concentration was determined by the method of Lowry et al. (Lowry et al. 1951) with bovine serum albumin (BSA) as the reference.

Results

Induction of dechlorination with different halophenols

The ability of strain 2CP-C to utilize halophenols as terminal electron acceptors had been previously reported, however it had not been established whether this activity was constitutive or induced. To address this question, cultures that were pregrown with fumarate as the terminal electron acceptor were challenged with a variety of halophenols. Dehalogenation occurred immediately after the addition of each halophenol, indicating the presence of constitutive activity (Table 1). The constitutive dechlorination rates remained constant for at least four hours and varied among the halophenols tested, with 2,6-DCP having the highest rate. To determine if dechlorination activity was inducible, and if different halophenols might exhibit different induction characteristics, inducing substrates were added to fumarate-grown cells in late log phase (OD 600 nm = 0.08). After inducer addition, 2-CP was added at 24 h and 48 h and dechlorination rates were measured. With the exception of 2,4-DCP the inducing substrates were completely dechlorinated at 24 h. From previous experiments the impact of dechlorination products such as phenol and 3-CP on 2-CP dechlorination rates had been shown to be minimal (data not shown). 2-CP was completely dechlorinated in less than 24 h after each addition and typical results of a single induction experiment are shown in Figure 1. Some induction of dechlorination activity was evident four hours after the addition of inducing substrate as indicated by the exponential increase in the dechlorination rates above the initial constitutive levels. The protein concentration at the beginning and end of induction experiments was on average 25.7 and 26.0 mg protein as BSA L^{-1}, respectively, indicating no significant increase in biomass occurred.

All halogenated phenols tested induced dechlorination activity (Table 2). For example, the constitutive 2-CP dechlorination rate of 3.56 μmol/h (0.138 μmol of Cl^- h^{-1} mg of protein^{-1}) was much lower than the induced rate of 31.2μmol/h (1.20 μmol of Cl^- h^{-1} mg of protein^{-1}).

Depending on the specific inducing substrate used, the measured dechlorination rates of 2-CP varied.

Table 1. Summary of constitutive *o*-dehalogenation rates of various halophenols in non-induced cultures.[a]

Substrate	Dehalogenation rates[b] (μmol of Cl^- h^{-1} mg of protein^{-1})
2,6-DCP	0.40 ± 0.12
2,5-DCP	0.09 ± 0.03
2,4-DCP	0.04 ± 0.01
2-BrP	0.07 ± 0.03
2-CP	0.14 ± 0.06

[a] Dehalogenation rates were calculated from the depletion of substrate added into replicate non-induced cultures of the same cell density. Rate calculations were based on the substrate dehalogenated within three hours from the addition of substrate.
[b] Reported are mean ± standard deviation based on triplicate cultures.

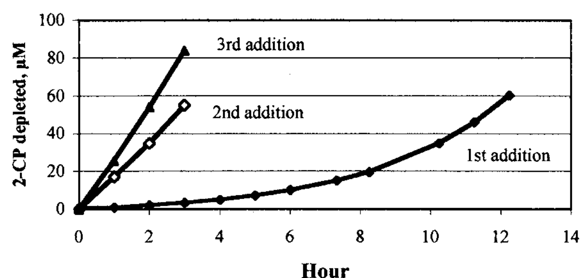

Figure 1. Typical dechlorination results from an induction experiment, in this case, 2-CP as the inducing substrate. The first 130 μM 2-CP was added to the non-induced culture during late log phase. The second and third additions of 130 μM 2-CP were made 24 and 48 hours after the first addition. The first three hours of data after the addition of 2-CP were used to calculate dechlorination rates.

Table 2. Summary of dechlorination rates (μmol of Cl^- h^{-1} mg of protein-1) of 2-CP following induction by various CPs in non-induced cultures.[a] Reported are mean ± standard deviation of triplicate cultures.

Inducing substrate	24 h after induction[b]	48 h after induction
2,6-DCP	1.18 ± 0.16	1.53 ± 0.06
2,5-DCP	0.69 ± 0.14	1.18 ± 0.12
2,4-DCP	0.21 ± 0.04[c]	0.49 ± 0.06
2-BrP	1.59 ± 0.08	2.26 ± 0.09
2-CP	0.80 ± 0.04	1.20 ± 0.07

[a] 130 μM of inducer was added at late log phase. 130 μM 2-CP was added 24 and 48 hours after the addition of inducing substrate.
[b] Dechlorination rates after induction were calculated from the initial depletion of 2-CP added 24 and 48 hours later.
[c] 2,4-DCP was not completely dechlorinated when 2-CP dechlorination rates were determined and is known to inhibit 2-CP dechlorination.

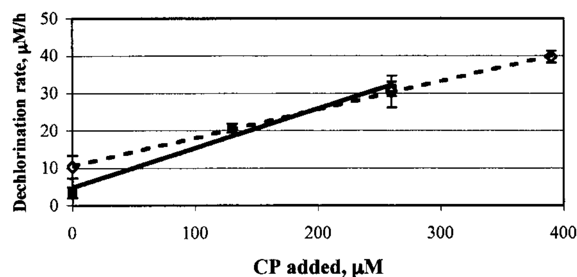

Figure 2. Relationship between the increase in dechlorination rates and the cumulative addition of 2-CP or 2,6-DCP reported as cumulative CP-chloride. Dechlorination rates were measured after the addition of different cumulative CP-chloride additions. The solid line is for a 2-CP fed culture and the dashed line is for a 2,6-DCP and 2-CP fed culture. Initial constitutive dechlorination rates for 2-CP and 2,6-DCP were used for no addition of CP. The lines are the result of linear regression with standard deviation error bars.

Most notably, the addition of 2-BrP resulted in much higher rates than exhibited with other halophenols tested, indicating the induction potential of 2-BrP was greater than even 2-CP (Table 2). The other inducers were ranked, based on the efficacy of induction, as follows: 2,6-DCP > 2-CP = 2,5-DCP > 2,4-DCP. The considerably lower dechlorination rate of 2-CP observed after inducing with 2,4-DCP was due to the presence of residual 2,4-DCP at 24 h. Prior experiments showed that dechlorination of 2,4-DCP to 4-CP was slow compared to other halophenols and that the presence of 2,4-DCP inhibited the dechlorination of 2-CP (data not shown).

Although biomass concentrations appeared unchanged during these experiments as monitored by the optical density and protein content, the increase in dechlorination rates was proportional to the amount of halophenols dechlorinated (Figure 2), which suggested the increased rates were associated with some new growth. This new growth was clearly negligible compared to the total concentration of cells in the experimental system, since the yield of biomass from 130 μM 2-CP is very small, approximately 50-fold less, relative to the yield from 5 mM fumarate. This could also explain the higher dechlorination rate observed after induction by 2,6-DCP since two *ortho*-chlorines instead of one are removed (i.e., 2,6-DCP is dechlorinated to phenol by strain 2CP-C) during the induction phase (first 24 hour) thus theoretically yielding twice the dechlorinating biomass.

Induction of dechlorination by phenol

Phenol also appeared to induce dechlorination. Non-induced cultures exposed to phenol had greater dechlorination rates compared to cultures not exposed to phenol (Table 3). This apparent increase in activity, however, diminished after the addition of more 2-CP and, after two more additions of 130 μM 2-CP the dechlorination rate of these phenol-exposed cultures were nearly identical to that of cultures not subjected to phenol.

Induction threshold concentration

To evaluate the minimum threshold concentration of 2-CP that would induce dechlorination, increasingly lower concentrations of 2-CP were added as inducing substrates during growth. Dechlorination rates above normal constitutive levels were observed at 2-CP addition concentrations of greater than 0.5 μM (Table 4). Concentrations at and below 0.5 μM showed no increase above constitutive dechlorination activity. After induction, however, rates varied such that the cultures that received the most 2-CP exhibited higher sustained dechlorination after induction. This was clear from the relative increase in dechlorination rate, calculated by dividing the measured rate by the total concentration of 2-CP added (Table 4). From this calculation, only the 5 and 50 μM 2-CP additions resulted in higher sustained dechlorination rates through two additions of 2-CP at 24 h and 48 h. As observed in the previous experiment, the dechlorination rates increased as these additional 2-CP spikes were added.

Utilization of 2-CP and fumarate in dense and dilute cultures

Since dechlorination rates in induced cells increased in proportion to the concentration of halophenol added, it appeared that dechlorination activity was not induced in most of the fumarate-grown biomass. To determine if this was the case, relative rates of dechlorination and fumarate utilization in dense and dilute fumarate-grown cultures were measured after induction of dechlorination activity. The hypothesis was if 2-CP dechlorination activity was induced in the majority of cells, the dilute culture would have a lower dechlorination rate than the concentrated culture, since the cell concentration would be much lower. Similarly, since the parent culture was grown on fumarate, the dilute culture would also have proportionally lower

Table 3. Increase of dechlorination activity by cells grown with phenol.[a]

Culture	2-CP dechlorination rate, μmol of Cl^- h^{-1} mg of protein^{-1c}		
	Late log growth	24 h	48 h
Grown with phenol[b]	0.54 ± 0.08	0.73 ± 0.05	1.22 ± 0.03
Non-induced	0.14 ± 0.05	0.80 ± 0.04	1.20 ± 0.07

[a] The first addition additions of 130 μM 2-CP were added at late log phase. The second and third additions of 130 μM 2-CP were added 24 and 48 hours after the first addition.
[b] Phenol was added initially at a concentration of 50 μM when cultures were first inoculated.
[c] Reported are means ± standard deviations on triplicate cultures.

Table 4. Influence of different concentrations of 2-CP on induction of dechlorination activity and on the relative increase in dechlorination rates after further 2-CP additions.[a]

2-CP added, μM	2-CP dechlorination rate, μmol of Cl^- h^{-1} mg of protein^{-1} [b]		Relative increase in dechlorination rate
	Initial rate	24 h	(μmol of Cl^- h^{-1} mg of protein^{-1})/μM added[c]
0	0.16 ± 0.05	0.81 ± 0.02	0.13
0.05	0.14 ± 0.08	0.76 ± 0.06	0.13
0.5	0.20 ± 0.04	0.76 ± 0.04	0.12
5	0.26 ± 0.05	0.95 ± 0.13	0.14
50	0.52 ± 0.10	1.52 ± 0.10	0.19

[a] 2-CP was added at various concentrations at inoculation along with 4 mM acetate and 5 mM fumarate. 130 μM 2-CP was added when cells reaching late log phase. The second addition of 130 μM 2-CP was added 24 hours after the first addition.
[b] Reported are mean ± standard deviation in triplicate cultures.
[c] Relative increase in dechlorination after 2-CP addition was normalized by dividing the dechlorination rate by the concentration of 2-CP added.

rates of fumarate reduction than the concentrated culture. As expected, the utilization rate of fumarate was 87 times lower in the diluted culture, approximately following the dilution ratio (1:100) (Table 5). In contrast, dechlorination rates of 2-CP in dense cultures and diluted cultures were nearly identical after induction of dechlorination with 260 μM of 2-CP (Table 5). The induction time for both dense and dilute dechlorinating cultures was similar. Succinate, the product of fumarate reduction, did not effect dechlorination of 2-CP (data not shown). The dense cultures had 25.73 ± 0.35 mg of protein/L and the corresponding protein- based specific dechlorination rate of 2-CP was 1.16 ± 0.04 μmol of Cl^- h^{-1} mg of protein^{-1}. Since a 1:100 dilution was used and little growth occurred with the 260 μM of 2-CP added, the total protein content remained relatively constant. Based on this, the dechlorination rate for dilute cultures was estimated as 116 μmol of Cl^- h^{-1} mg of protein^{-1}, or 100 times the rate in dense cultures. In contrast, the protein-based specific utilization rates of fumarate were approximately the same between dense and dilute cultures (Table 5).

Discussion

All of the *ortho*-chlorophenols tested in this study induce dechlorination activity, and were themselves dechlorinated (Table 1). The induced dehalogenase appears to be the same for all halophenols tested, since the cultures induced by individual compounds all readily dechlorinate 2-CP. This is not surprising as induction of dechlorinating activity is widely reported among chloroaromatic compound-respiring microorganisms (Madsen & Aamand 1992; Mohn & Kennedy 1992; Bouchard et al. 1994; Cole et al. 1994; Utkin et al. 1995; Löffler et al. 1996; Sanford et al. 1996; Dennie et al. 1998; Sun et al. 2000). Some of these chlororespiring bacteria are also able to remove *ortho*-substituted halogens from phenolic compounds. The reductive dehalogenase of one of these bacteria, *Desulfitobacterium dehalogenans*, was recently purified

Table 5. Comparison of utilization rates of 2-CP and fumarate in dense and dilute cultures.

Culture	Electron acceptor	Electron acceptor utilization rate, μM/hour[c]	Ratio of utilization rates, dense/dilute (expected)[e]
Dense[a]	2-CP	30.56 ± 2.01[d]	1.02 (100)
Dilute[b]	2-CP	29.95 ± 0.79[d]	
Dense	Fumarate	328.88 ± 24.24	87.70 (100)
Dilute	Fumarate	3.75 ± 0.07	

[a] Dense cultures were grown to late log phase with 5 mM fumarate and 2 mM acetate before 2-CP or additional fumarate were added.
[b] Dilute cultures were obtained by 100-fold dilution of dense culture in fresh media.
[c] Mean $\pm$ standard deviation. The results are based on triplicate cultures.
[d] Dechlorination rates of 2-CP were measured after induction by two 130 μM additions of 2-CP in non-induced dense and dilute cultures.
[e] Calculated by the ratio of rates for dense:dilute cultures. Expected values are equal to dilution factor.

and characterized (van de Pas et al. 1999). It is possible that strain 2CP-C may have a similar dehalogenase, however the substrate specificity for these two bacteria are quite different (Utkin et al. 1995).

In our experiments significantly greater dechlorination rates of 2-CP were achieved after induction by 2-BrP, compared to the activity induced by other halophenols. This is a somewhat interesting result since 2-BrP was not the original substrate for dehalogenation and bromide is not abundant in the terrestrial environments from where all *Anaeromyxobacter* strains have been isolated.. The likely explanation for this is that 2-BrP is a better inducing substrate than the other CPs tested, yielding higher rates of dechlorination. As a result induced cells have more dehalogenase per unit biomass and increased dechlorination activity. Although it might be expected, a difference in energetic yield between 2-BrP and 2-CP is not likely, since thermodynamically the difference between dechlorination and debromination is very small (e.g., -145.4 vs. -146.5 kJ/reaction for 2-chlorobenzoate and 2-bromobenzoate respectively) (Dolfing & Harrison 1992). Regardless of the explanation, this type of phenomenon has not been previously reported in the literature for halorespiring microorganisms. It illustrates the important effects of different inducing substrates and particularly reveals the potential role of brominated organics for inducing activity. This could clearly have a potential benefit for enhancing the rates of dehalogenation in bioremediation. For example, it would be interesting to test 2,6-dibromophenol as an inducer, to stimulate or even increase dechlorination activity. Also it would be beneficial to test other halorespiring organisms to see if they respond in a similar fashion to brominated compounds.

Phenol appears to be a poor inducer relative to the other halophenols tested. Since phenol was added at the beginning of inoculation and was not metabolised by strain 2CP-C, it persists during growth on acetate and fumarate. The induced dechlorination activity associated with phenol does not persist, since dechlorination rates are identical with 2- CP-induced cultures without phenol when more 2-CP is added to the cultures. This suggests that halophenols potentially displace phenol as inducing substrates and perhaps are better inducers of dechlorination. More study is needed to elucidate the nature of this phenomenon.

The concentration of halophenols that triggers a shift from constitutive to induced dechlorination activity is also of interest. It has been recognized that a threshold concentration exists in the induction of enzymes responsible for biodegradation and biotransformation of many environmental contaminants (Alexander 1999). Little information, however, is available in the literature about the induction threshold concentration of reductive dechlorination in halorespiring bacteria. Our study shows that this threshold concentration for 2-CP is around 5 μM, as no increase above constitutive activity levels were observed below this concentration (Table 4). This is similar to threshold concentrations obtained for the degradation of chlorobenzoate and phenylamide herbicides (Reber 1982; Lechner & Strauble 1984). The relative increase in dechlorination rates that occurred with the 5 and 50 μM additions of 2-CP yielded sustained higher dechlorination rates, suggesting that exposing fumarate-growing cells to 2-CP has some benefit in terms of dechlorination activity. A low induction concentration of 2-CP and the presence of significant constitutive activity suggests the possibility that

Table 6. Comparison of dechlorination rates between selected halorespiring bacteria.

Strain	Best substrate[a]	Dechlorination rate μM Cl^- h^{-1} mg protein^{-1}	Reference
Anaeromyxobacter dehalogenans 2CP-C	2-CP	116.1 ± 4.2[b]	This study
Desulfomonile tiedjei DCB-1	3-CB	1.1	19
Desulfitobacterium hafniense DCB-2	2,4,6-TCP	1.8	15
Desulfitobacterium chlororespirans Co23	3-Cl-4-HBA	6.88 ± 2.18[c]	24
Desulfitobacterium dehalogenans JW/IU-DC1	2,3-DCP	11.6[c]	31
Dehalospirillum multivorans	PCE	3	26
Dehalococcoides ethenogenes strain 195	PCE	4.14 ± 0.63	16
Isolate MS-1	PCE	1.0[c]	27

[a] The substrate with the highest rate reported in the cited reference. 2-CP was the only substrate tested for *A. dehalogenans* strain 2CP-C. Abbreviations: 2-CP for 2-chlorophenol; 3-CB stands for 2-chlorobenzoate; 2,4,6-TCP for 2,4,6-trichlorophenol; 3-Cl-4-HBA stands for 3-chloro-4-hydroxybenzoate; 2,3-DCP for 2,3-dichlorophenol; PCE for perchloroethene.
[b] Dechlorination rate in dilute cultures were estimated by multiplying the dense culture rate by the dilution factor of 100.
[c] Cellular protein content is assumed to be 50% of the dry biomass.

very low concentrations of environmental contaminants may be amenable to bioremediation even when non-induced cells are used.

Although dechlorination activity is induced by several halophenols in *A. dehalogenans* strain 2CP-C, this activity is not induced in the majority of biomass. This is particularly evident when comparing the utilization rates of 2-CP and fumarate in dense and dilute cultures (Table 5). The substrate utilization rate in dilute cultures was expected to be 100-fold less than that in dense cultures, with either fumarate or 2-CP. With fumarate this does occur, however with 2-CP, the dechlorination rate after induction is the same in dense and dilute cultures. Apparently, the majority of the biomass in the dense cultures contributes very little to the dechlorination capacity of the culture. This suggests that only the new cells growing by chlororespiration of 2-CP are responsible for dechlorination activity, while the pre-existing fumarate-grown cells are not induced to dechlorinate. This is also supported by the observation of proportional increases in dechlorination rates with the amount of halophenols added to the cultures (Figure 2). Since this occurred regardless whether dense or dilute cultures were used, it is clear additional research needs to be done to explain this physiological phenomenon.

Halorespiring bacteria have been found worldwide and likely occur in diverse habitats. For example the myxobacteria, and probably *Anaeromyxobacter dehalogenans*, are known to have wide global distribution (Reichenbach & Dworkin 1992; Dawid 2000). As a result, it is likely that these bacteria participate in the natural attenuation of haloorganic compounds such as chlorophenols and are good candidates for use in bioaugmentation. If, however, induced dechlorination only occurs in new cell growth, as it did apparently with strain 2CP-C, the distribution of this behavior is potentially very important. Since there has been considerable interest in using halorespiring bacteria for bioaugmentation and bioremediation of sites contaminated with chlorinated compounds, the physiological state of such cultures could be very important in determining whether a process will work or not. For example, a common strategy employed in bioaugmentation of halogenated compounds is to apply active biomass (Mikesell & Boyd 1988; Christiansen & Ahring 1996; Miethling & Karlson 1996; El Fantroussi et al. 1997). If the active dechlorinating biomass is only proportional to the chlorinated substrate added, the use of a non-chlorinated growth substrates (e.g., fumarate or pyruvate) to obtain a large amount of biomass would not be a very useful strategy.

Dechlorination kinetic studies also typically involve cultivation of cells using a readily utilized substrate, such as pyruvate or fumarate, followed by the addition of a halogenated substrate to induce activity. This is done because specific rates based on biomass are measured more easily with a large number of cells. Based on our findings, this strategy is not valid since only a small fraction of the resulting biomass actively dechlorinates the substrate. This suggests that previous results of dechlorination rate studies and process

evaluations might be misleading and actually underestimate the actual activity. Admittedly, this should be confirmed in other halorespiring bacteria, however, it is a reasonable hypothesis that they would behave similarly to strain 2CP-C. For example in our study, the dechlorination rate measured in dense cultures after induction was within same order of magnitude to rates reported in other studies (Table 6). Since the dechlorination rate remained the same in dilute cultures of strain 2CP-C with 100-fold less biomass, the actual dechlorination activity of the culture is actually much higher than any rate previously reported for any chlororespiring organism.

Future studies with this organism may provide a new perspective in the research of chlororespiring bacteria and their use in certain environmental applications. *Anaeromyxobacter dehalogenans* strains are also attractive as candidates for further research because of the rapid dechlorination rates in dilute cultures which shortens the duration of routine experiments to hours instead of days, as is the case with other isolates. This makes it an ideal model organism for research on reductive dechlorination of chlorophenols and chlororespiration in general.

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