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Biodegradation of 2-chlorobenzoate by recombinant *Burkholderia cepacia* expressing *Vitreoscilla* hemoglobin under variable levels of oxygen availability

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Abstract

The influence of bacterial hemoglobin, VHb, on dechlorination and degradation of 2-chlorobenzoate (2-CBA) by recombinant *Burkholderia* sp. under variable oxygen availability with an initial dissolved oxygen concentration of 0.27 mM-0.72 mM was investigated in batch and continuous culture. Ability to express VHb was provided to recombinant *Burkholderia* by transformation with the VHb gene, *vgb*, on plasmid pSC160. 100% of 0.5 mM CBA was degraded in cultures with 85% and 70% of total volume as headspace air in closed reactors by both wild type and recombinant *Burkholderia*. The recombinant cultures were able to dechlorinate and degrade 100% of the 2-CBA in less than 48 hours at 30 °C compared to more than 120 hours for wild type cultures. The rate and extent of CBA degradation by recombinant cultures with 40% of total volume as headspace air was higher than those achieved by wild type cells at the end of the 168 hours of incubation period, 98 and 73%, respectively. The chloride released: CBA degraded molar ratio for cultures with 40% of total volume headspace air was nearly stoichiometric (molar ratio = 1.0) for recombinant strains, whereas it was non-stoichiometric (molar ratio = 0.24) for wild type cells. The results suggest a suicidal *meta*-pathway for wild type cells and a complete dechlorination and degradation pathway for recombinant cells under hypoxic conditions. The degradation and dechlorination ability of both types of cells was also investigated in continuous reactor studies by varying the dilution rate under hypoxic conditions. Regarding potential of the recombinant strain for 2-CBA degradation in either open ecosystems or closed bioreactor bioremediation systems, the stability of the plasmid containing *vgb* in the recombinant cells was also studied; the plasmid was 100% stable at 0.025 h^{-1} dilution rate (∼1.7 d hydraulic retention time), even after one month.

Introduction

The release of chlorinated organic compounds into the environment is a widespread concern due to their toxicity to humans and wildlife, their relative persistence in aquatic sediments and soils, and their bioaccumulation (Niedan & Schöler 1997; Deewerd & Bedard 1999). Chlorinated benzoic acids (CBA) have been intentionally released to the environment due to their use in agriculture as herbicides or pesticides, or unintentionally because they are common metabolites in the aerobic transformation of many chlorinated pollutants, including polychlorinated biphenyls (PCBs), and alkyl benzenes (Krooneman et al. 1996; Flanagan & May 1993; Van der Wouede et al. 1995). Recently, Niedan & Schöler (1997) have also reported abiotic formation of CBAs from naturally occurring compounds, such as trichloroacetic acid. 2-CBA is difficult to degrade by microorganisms because of the *ortho*-position of the chlorine (Van der Wouede et al. 1996). 2-CBA also constitutes a large portion (∼55–60%) of observed metabolites of aerobic PCB degradation in the contaminated upper Hudson River sediments (Flanagan &

May 1993; Harkness et al. 1993). Like most chlorinated aromatics, degradation of 2-CBA occurs by cometabolism. The 2-CBA concentrations that are below the toxic levels are less than the required amount of carbon and energy source to maintain microbial biomass within the system or to provide energy for rate limiting reactions (Champagne et al. 1998).

At least four different aerobic biodegradation pathways mineralizing CBA, and a fifth pathway in *Alcaligenes* sp. strain L6 (via gentisate and not involving (chloro) catechol) have been identified (Krooneman et al. 1996). The first step in the *ortho*-dehalogenation pathway is dihydroxylation mediated by *ortho*-CBA 1,2-dioxygenase, followed by dehalogenation. This results in conversion of 2-CBA to catechol, which is readily metabolized to muconate (Romanov & Hausinger 1994). In the modified ortho-cleavage pathway 2-CBA is oxidized by chlorocatechol 1,2-dioxygenase. *Ortho*-cleavage of the resulting chlorocatechol produces chloro-*cis,cis*-muconates; this is followed by cycloisomerization accompanied by chloride release (Schmidt & Knackmuss 1980; Vollmer & Schlömann 1995). If *meta*-cleavage takes place, 2-CBA is transformed to 3-chlorocatechol. Subsequent production of the acylchloride from 3-chlorocatechol by catechol 2,3-dioxygenase leads to inactivation of catechol dioxygenases (Bartels et al. 1984; Arensdorf & Focht 1994). Recently Mars et al. (1997) have showed that *P. putida* strain GJ31 has a unique ability to degrade 3-chlorocatechol to 2-hydroxy-6-chloro-carbonyl-muconate, and then to 2-hydroxy-*cis*, *cis*-muconate accompanied with chloride release. This occurs via a *meta*-cleavage pathway, which includes a special catechol 2,3-dioxygenase. Thus, the *ortho*-dehalogenation, modified *ortho*, and gentisate pathways as well as the unique *meta*-cleavage pathway found in *P. putida* strain GJ31 will release one mole of chloride for every mole of 2-CBA metabolized. In the *meta*-cleavage pathway, however, most of the 2-CBA will be metabolized to an intermediate, which still contains chlorine in the ring, so there will be less than one mole of chloride released for each mole of 2-CBA disappearance.

Alcaligenes sp., *Pseudomonas* sp., *Rhodococcus* sp., *Acinetobacter* sp., and *Burkholderia* sp. are among the most commonly reported and extensively studied bacteria known to degrade CBAs (Arensdorf & Focht 1994, 1995; Tros et al. 1996; Krooneman et al. 1996; Zaitsev et al. 1984, 1995; Tsoi et al. 1999). *Burkholderia* sp. have been reported to be particularly able to oxidize a wide range of PCBs and CBAs (Seeger et al. 1999; Arensdorf & Focht 1995; Zaitsev & Karasevich 1984). This ability is strongly dependent on the availability of oxygen in the medium containing CBA (Krooneman et al. 1999, 1996; Viliesid & Lilly 1992). This is because oxygen is required not only as a terminal electron acceptor in the respiratory electron transfer chain but also as a substrate in the degradation-pathways (Krooneman et al. 1996; Tsoi et al. 1999). In soils and sediments contaminated with PCBs, oxygen availability is poor due to the mass transfer limitations in the porous media. Therefore, oxygen supply to the bacteria must be enhanced by engineering methods that overcome mass transfer limitations and/or genetic engineering of bacteria to grow and metabolize the substrate under hypoxic conditions.

Wakabayashi et al. (1986) reported the presence of a dimeric hemoglobin (VHb) in the obligately aerobic bacterium *Vitreoscilla* which allows it to survive and grow better under hypoxic conditions. VHb synthesis is increased by about 10-fold when the dissolved oxygen (DO) concentration is less than 5% of air saturation (Joshi & Dikshit 1994). It has been demonstrated that VHb has a cytoplasmic localization adjacent to the cell membrane (Ramandeep et al. 2001), which enables the bacteria to survive in oxygen-limited environments probably by acting as an 'oxygen storage trap' and facilitating oxygen delivery to terminal oxidases (Webster 1987; Park et al. 2002).

The gene (*vgb*) encoding VHb has been isolated, sequenced, characterized, and successfully cloned into heterologous hosts such as *E. coli* (Dikshit & Webster 1988; Khosla & Bailey 1988a). The engineering of *Burkholderia* strain DNT with *vgb* was shown to enhance degradation of 2,4-dinitrotoluene (Patel et al. 2000; Nasr et al. 2001; Chung et al. 2001). In this case a VHb-mediated increase in intracellular O_2 may occur, in turn leading to increased growth (Patel et al. 2000; Nasr et al. 2001), increased production of enzymes in the catabolic pathway (Fish et al. 2000), or, perhaps, increased delivery of O_2 to the oxygenases among them (Lin et al., in press). Thus, VHb technology may be useful in enhancing degradation of haloaromatic compounds in the environment when oxygen-poor conditions exist in contaminated sites.

The focus of this paper is to extend VHb technology to the enhancement of degradation of 2-CBA by *Burkholderia*, especially under hypoxic conditions. The effects of aeration in batch cultures were studied by varying the ratio of volume of culture medium to total flask volume. The degradation and dechlor-

ination ability of both *vgb*-containing and *vgb*-free cells was investigated in continuous reactor studies by varying the dilution rate. The use of the recombinant strain for 2-CBA degradation in either open ecosystems or closed bioreactor systems depends on the ability to retain the *vgb*-containing plasmid over the long periods needed for biodegradation. Hence, plasmid stability in *Burkholderia* was also investigated during this research.

Materials and methods

Microorganisms and plasmid

Burkholderia sp. strain DNT (Spanggord et al. 1991) and genetically engineered *Burkholderia* transformed with plasmid pSC160 (Patel et al. 2000) were provided by Drs. DA Webster & BC Stark (Illinois Institute of Technology, BCPS Department). Plasmid pSC160 (16.0 kb) contains *vgb* and confers resistance to both ampicillin and kanamycin (Liu et al. 1995).

Media and growth conditions

The *Burkholderia* strains were maintained at 30 °C on Luria-Bertani (LB) agar plates; the strain with pSC160 was grown on plates supplemented with 100 μg/ml ampicillin and 40 μg/ml kanamycin. The composition of the chloride-free mineral salts medium has been described by Zaitsev et al. (1995), and contained 0.5 mM 2-CBA (Sigma-Aldrich, WI, USA; 98% purity) and 10 mM acetate (Fisher, IL, USA) as substrates. For batch and continuous reactor experiments, the cells were pre-grown in LB medium supplemented with 0.5 mM CBA (and antibiotics for the recombinant strain), up to late exponential phase, and then harvested by centrifugation (10,000 $\times$ *g*), washed with chloride-free mineral salts medium and resuspended in the same medium. A 1% (v/v) inoculum was used to inoculate the reactors. Both plasmid-free wild type and recombinant cells were grown without antibiotic pressure during this period to provide information necessary on the inherent stability of pSC160.

Determination of volumetric mass transfer coefficient (K_La) and oxygen transfer rate (OTR) in the culture medium

The determination of K_La and OTR values in the culture medium at 30 °C was performed according to Rainer et al. (1990). The dissolved oxygen was measured with a YSI 5100 DO meter (Yellow Springs, OH, USA) and a DO probe attached to an impeller inserted into the flasks or reactor vessel, which was tightly sealed to prevent oxygen transfer from the outside. Zero percent oxygen saturation was obtained by sparging nitrogen into the sterilized medium. The full oxygen scale was established by opening the seal of flasks and agitating at 50 rpm, so that the medium is saturated with oxygen.

Batch experiments

The batch experiments with wild type and recombinant cells were conducted in triplicate at 30 °C in 150 ml of sterilized mineral salts medium in either 250 ml, 500 ml or 1,000 ml flasks; this corresponds to 40%, 70% and 85% of total volume as head space air, respectively. 2 ml samples were collected daily from each flask for seven days and used for further analysis (see below).

Continuous stirred reactor experiments

Continuous cultivation was performed in 2 liter chemostat reactors (containing 1.5 liter of mineral salts medium) which corresponds to 25% of total volume as head space air. The reactors were stirred at 50 rpm and the temperature was 30 °C. No additional air or oxygen was supplied to the reactor in order to maintain hypoxic conditions. 10 ml samples were collected daily from the reactor for 8–10 days and used for further analysis (see below).

Growth measurement

Viable cell counts were determined by serial dilution of samples followed by plating on LB plates for both strains. Plates were counted after incubation at 30 °C for 24 hours.

Analytical procedures

2-CBA was detected by HPLC as described by Tsoi et al. (1999). Samples for HPLC analysis were centrifuged and the supernatants filtered through a 0.2 μm filter. The analysis was performed with a 250 mm by 4 mm C_{18} column (Varian, Walnut Creek, CA, USA) with the mobile phase consisting of a mixture of 0.1% H_3PO_4 and acetonitrile (1 : 1) applied at a flow rate of 1.5 ml min^{-1}.

Table 1. k_La and OTR values as a function of percent headspace. Each data point is the average of measurements from three independent experiments and the error bars represent one standard deviation

Aeration mode	k_La (h^{-1})	OTR (mg l^{-1} h^{-1})
40% headspace	6.3 ± 0.2	1.63 ± 0.04
70% headspace	10.6 ± 0.3	2.75 ± 0.07
85% headspace	12.3 ± 0.4	3.20 ± 0.10

The dechlorination activity of the cells was determined after the centrifugation and filtration of culture medium. The release of chloride ions was measured using a chloride determination kit (effective in the range of 0–20 mg/l (0–0.56 mM) of free Cl^- with $\pm$0.6 mg/l accuracy) following the manufacturer's instructions (HACH Chemical Company, Loveland, CO, USA). Dechlorination activity was calculated based on the chloride mass balance in the culture medium before and after the cell growth.

Plasmid stability test

The percentage of plasmid carrying cells was determined by the ability to form colonies on ampicillin and kanamycin supplemented plates. Total viable cell counts were made on antibiotic-free LB agar plates. 100–200 colonies were randomly transferred with sterile toothpicks from each of these plates to LB agar plates containing 100 μg/ml ampicillin and 40 μg/ml kanamycin. These colonies were counted after incubation at 30 °C for 24 h.

Results and discussion

Batch experiments

DO concentration in the batch cultivation depends on the culture medium volume to flask volume ratio. The k_La and OTR values determined at 40, 70 and 85% of total volume as headspace air are given in Table 1. As expected, both parameters increased as the percent of headspace air increased.

The knowledge of the oxygen consumption profiles of wild type and recombinant cells is of importance to better understand their ability to degrade 2-CBA under variable oxygen availability. As seen in Figure 1, DO concentration profiles of both strains followed the same trend such that DO levels decreased

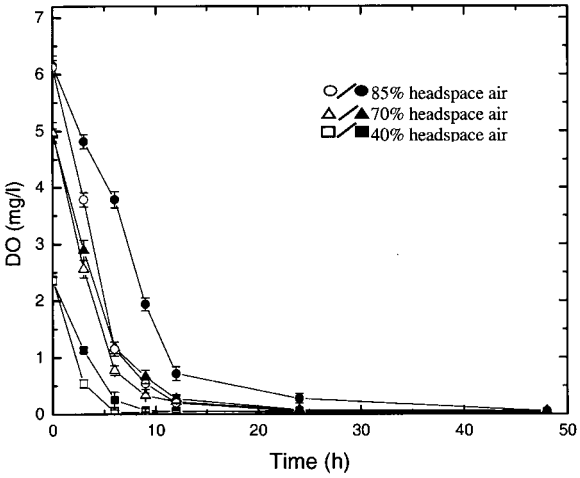

Figure 1. Dissolved oxygen concentration profiles of wild type (closed symbols) and recombinant cells (open symbols) in batch culture with various percent headspaces. Each data point is the average of three independent experiments and the error bars represent one standard deviation.

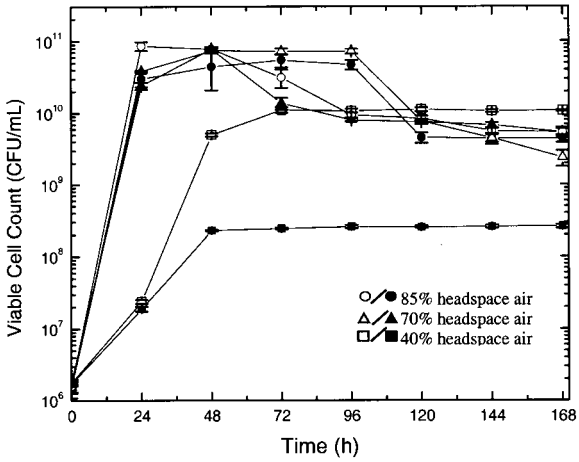

Figure 2. Comparative growth of wild type (closed symbols) and recombinant cells (open symbols) in batch culture. Each data point is the average of three independent experiments and the error bars represent one standard deviation.

with increasing cell densities in the culture medium. In all cases the recombinant cultures used up the oxygen more rapidly than the wild type cultures, presumably due to the faster growth of the former. DO eventually dropped to zero in all cases. This was especially true at 40% headspace, in which DO fell below 2% of air saturation at the beginning of exponential phase for both strains. Under these conditions, the recombinant cells fared much better than wild type cells regarding maximum viable cell counts (Figure 2) and doubling time (Table 2).

Table 2. Doubling times of wild type and recombinant *Burkholderia* in batch cultures

Aeration mode and strain	Doubling time, t_d (h)
40% headspace, wild type	9.31 ± 0.13*
40% headspace, recombinant	5.43 ± 0.11*
70% headspace, wild type	3.30 ± 0.17*
70% headspace, recombinant	1.84 ± 0.09*
85% headspace, wild type	1.72 ± 0.06
85% headspace, recombinant	1.52 ± 0.04

Each data point is an average of measurements from three experiments and the error bars represent one standard deviation. For each headspace condition an asterisk indicates that the wild type and recombinant values for that condition are statistically different at 95% confidence interval (t-test) with $P = 0.004$.

The enhancement of growth rate in batch cultures degrading 2-CBA due to the presence of *vgb* was seen under various aeration conditions (Table 2). The doubling times observed on 2-CBA were shorter than those reported for other strains capable of growing on various chlorinated organic compounds, including 2-CBA (Zaitsev et al. 1995; Oldenhius et al. 1989; Oltmanns et al. 1988; Reineke et al. 1984; Balajee & Mahadevan 1990; Kröckel et al. 1987). It can be seen from Table 2 that the *vgb*-bearing strain does not have any advantage over the wild type at high aeration (85% headspace air). At both 40 and 70% headspace air, the recombinant strain had a significantly lower doubling time than the wild type strain. This is presumably due to the presence and expression of *vgb*/VHb in the recombinant strains under low aeration conditions (Dikshit et al. 1990).

Figure 2 shows the growth curves of wild type and recombinant *Burkholderia* from the batch culture experiments under various aeration conditions. The maximum viable cell counts were similar at the higher aeration conditions (70% and 85% head space air) for both strains, but much higher for the recombinant strain compared to the wild type strain under the lowest aeration (40% head space air).

The better growth of *vgb*-bearing *Burkholderia* on 2-CBA containing medium is consistent with a number of studies in which the presence of *vgb*/VHb has been shown to enhance bacterial growth (Dikshit & Webster 1988; Khosla & Bailey 1988b; Patel et al. 2000). As mentioned above, this is likely due to enhancement of respiration by VHb (Webster 1987; Tsai et al. 1996; Park et al. 2002), which results in more rapid conversion of substrate to energy and new cell mass.

Figures 3 and 4 show the 2-CBA biodegradation/transformation and dechlorination ability of both strains in culture medium containing 0.5 mM 2-CBA. Rates of disappearance of 2-CBA for wild type cells occurred in the order 85% > 70% > 40% headspace air. The release of free chloride into the culture medium under the three conditions occurred in the same order (Figure 3). Table 3 shows the molar ratios of chloride release to 2-CBA degraded as a function of time for all three aeration conditions for wild type cells, and Table 4 shows the same results under the same conditions for the recombinant strains. It can be seen that under high aeration conditions (85% headspace air), the chloride released to 2-CBA degraded molar ratio is 1.0, indicating stoichiometric equivalence. Similar results are seen at 70% headspace air where the molar ratios indicate a nearly stoichiometric relationship between chloride release and 2-CBA degradation. At 40% headspace air, however, the average molar ratio is only 0.24 for wild type cells indicating that 2-CBA degradation/transformation was not accompanied by the stoichiometric release of chloride. Based on the suggested pathways for 2-CBA degradation previously discussed, non-stoichiometric chloride release (compared to 2-CBA disappearance) is explained only by the *meta*-cleavage pathway but not by the *ortho*-dehalogenation, modified *ortho* or unique *meta*-cleavage pathways (Romanav & Hausinger 1994; Schmidt & Knackmuss 1980; Vollmer & Schlömann 1995; Mars et al. 1997). In the case of recombinant cells at 40% headspace air, chloride release is nearly stoichiometric with 2-CBA disappearance. One possibility is that *Burkholderia* has more than one pathway for 2-CBA degradation, an *ortho*-dehalonogenation, modified *ortho*, or unique *meta*-cleavage pathway which is used when oxygen is relatively available and a suicidal *meta*-cleavage pathway that is used when oxygen is relatively limiting. Both *ortho*- and *meta*-cleavage pathways for chloroaromatic compound degradation are known to exist together in several soil bacteria (Müller et al. 1996). The oxygen limitation may occur only under 40% headspace air conditions for the wild type strain in our experiments. At 40% headspace air for the recombinant strain, oxygen levels in the medium are actually lower than that for the wild type strain, but we presume that the presence of VHb in the recombinant strain may still be able to provide sufficient

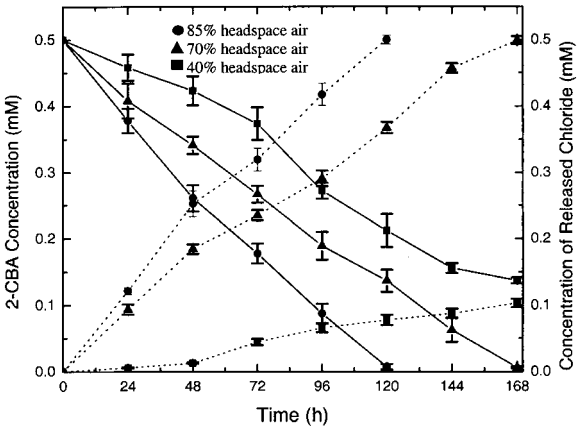

Figure 3. 2-CBA degradation (solid lines) and chloride release ((dashed lines) by wild type *Burkholderia* in batch culture. Each data point is the average of three independent experiments and the error bars represent one standard deviation.

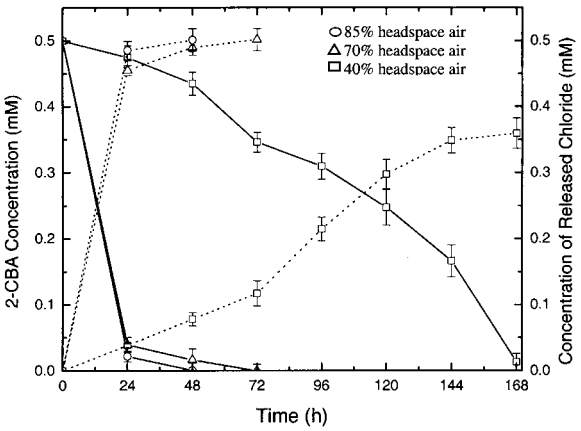

Figure 4. 2-CBA degradation (solid lines) and chloride release (dashed lines) by recombinant *Burkholderia* in batch culture. Each data point is the average of three independent experiments and the error bars represent one standard deviation.

Table 3. Stoichiometric differences between 2-CBA degradation and chloride release by wild type *Burkholderia* in batch cultures with variable percent headspace. Values at each time point are cumulative from $t = 0$ to that time

Time	(Released Cl^-)/(Degraded 2-CBA)		
(h)	40% headspace	70% headspace	85% headspace
0	0.0	0.0	0.0
24	0.15	0.77	1.00
48	0.17	0.92	1.00
72	0.25	0.96	0.99
96	0.29	0.96	1.00
120	0.27	0.98	1.00
144	0.25	1.00	
168	0.28	1.00	
Average	0.24	0.94	1.00

Table 4. Stoichiometric differences between 2-CBA degradation and chloride release by recombinant *Burkholderia* in batch cultures with variable percent headspace. Values at each time point are cumulative from $t = 0$ to that time

Time	(Released Cl^-)/(Degraded 2-CBA)		
(h)	40% headspace	70% headspace	85% headspace
0	0.0	0.0	0.0
24	0.75	0.0	1.00
48	0.84	0.94	1.00
72	0.76	1.00	
96	0.88		
120	1.00		
144	1.00		
168	0.74		
Average	0.86	0.97	1.00

oxygen to the cells to prevent the switch to the suicidal *meta*-pathway.

Even under high aeration (85% headspace air) conditions, 2-CBA degradation and chloride release to the medium are better for recombinant cells than wild type cells (Figures 3 and 4). This suggests that VHb enhances 2-CBA degradation even though the growth rates and maximum viable cells achieved are similar (Figure 2) and oxygen is presumably sufficient to support pathway(s) in which chloride is released stoichiometrically from 2-CBA (Table 3 and 4). Presumably, even under these conditions VHb supplies additional oxygen to the cells. Not surprisingly, the same trends occur with intermediate aeration (70% headspace air).

The approximate cell yields of recombinant cells at 70 and 85% headspace air were 1.5×10^{11} and 1.7×10^{11} CFU/ml per mM of degraded 2-CBA, respectively. The cell yields of wild type cells under these conditions were 1.1×10^{11} and 1.6×10^{11} CFU/ml per mM of degraded 2-CBA, respectively. Although, the cell yields of both strains are close to each other under these conditions, 2-CBA degradation by recombinant cells was much faster than that of wild type cells (Figures 3 and 4). At low aeration (40% headspace air), the recombinant strain has the greatest advantage in cell yield compared to the wild type strain, 2.3×10^9 CFU/ml per mM of degraded 2-CBA versus 1.4×10^6 CFU/ml per mM. This is presumably due

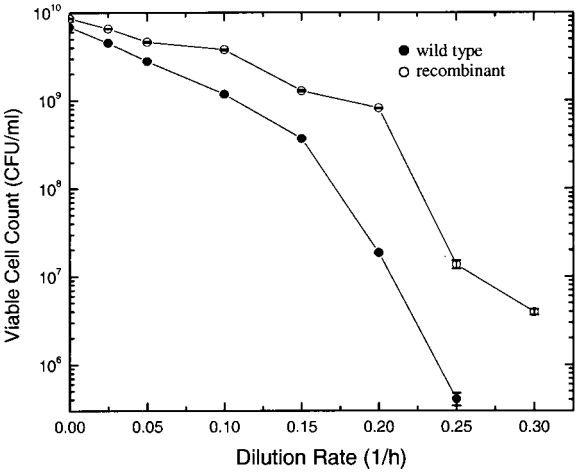

Figure 5. Growth of wild type (closed symbols) and recombinant (open symbols) *Burkholderia* cells in continuous culture. Wild type cells washed out after 0.25 h^{-1} dilution rate. Each data point represents the average of 5 measurements at steady state at that dilution rate, and the error bars represent one standard deviation.

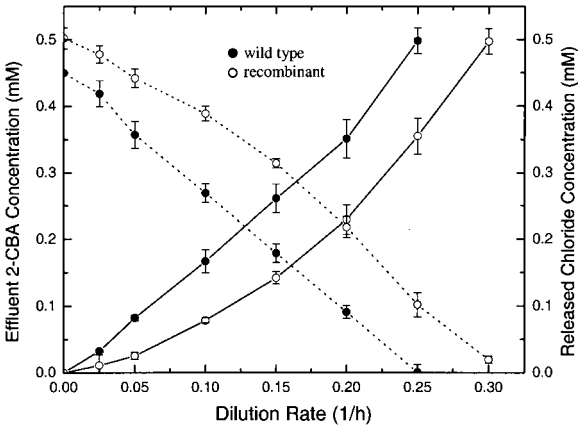

Figure 6. 2-CBA degradation (dark lines) and chloride release (dashed lines) by wild type and recombinant *Burkholderia* in continuous culture at various dilution rates. Each data point represents the average of 5 independent measurements at steady state at that dilution rate, and the error bars represent one standard deviation.

to the greater advantage afforded by *vgb*/VHb under hypoxic conditions.

Continuous culture experiments

The wild type and recombinant *Burkholderia* strains were grown with 25% headspace air, with no additional air or oxygen supplied to the chemostat (except that dissolved in the sterilized feed) in order to maintain hypoxic conditions. DO concentration in the feed was 2.5 ± 0.2 mg/l (0.28 mM). Ten hours after inoculation, DO dropped to zero and remained constant, indicating that the oxygen supply was not sufficient. The maximum specific growth rates of both strains during the batch cultivation were between 0.06 and 0.44 h^{-1}, depending on aeration mode. Therefore, dilution rates were kept below 0.44 h^{-1} in the continuous cultivation mode. Figure 5 shows the steady state viable cell counts of wild type and recombinant cells in the continuous culture reactors at various dilution rates. Although wild type cells washed out from the system beyond 0.25 h^{-1} dilution rate, recombinant cells were still stable up to 0.30 h^{-1} dilution rate. Comparison of the viable cell counts at steady state in the reactors indicates that recombinant cell production was higher than that of wild type cells at each dilution rate. As with the batch experiments, this is presumably due to the positive effects of VHb on respiration and 2-CBA metabolism.

The steady state effluent 2-CBA and free chloride concentrations at various dilution rates are shown in Figure 6. At higher dilution rates, 2-CBA was not degraded well by either strain as indicated by high effluent CBA and low chloride concentrations. The degradation ability of recombinant cells, however, was higher than that of wild type cells at each dilution rate as indicated by significantly lower effluent 2-CBA concentration and higher free chloride concentration. These results indicate that the *vgb*-containing recombinant cells have an advantage over wild type cells in terms of CBA degradation in continuous culture as well. Again, this is presumably due to the positive effects of VHb under hypoxic conditions.

The use of VHb technology for degradation of chlorinated or other aromatic compounds in either open ecosystems or closed bioreactor bioremediation systems rests on the ability of the recombinant cells to retain and express *vgb* in long-term (degradation time) operations. However, very little is known about the stability of pSC160 in engineered *Burkholderia* in the bioreactor systems. In order to determine its fate under hypoxic conditions, plasmid stability experiments were performed at 0.025 h^{-1} dilution rate for one month. The stability of plasmid pSC160, determined by retention of resistance to both ampicillin and kanamycin during cell growth, was 100% over the entire period. This stability was maintained in the absence of selective antibiotic pressure, which suggests the feasibility of long-term use of *vgb*-containing recombinant cells in the bioremediation of toxic compounds in engineered systems, particularly under hypoxic conditions.

Conclusions

The presence of *vgb*/VHb in *Burkholderia* enhanced growth and metabolism of 2-CBA under a variety of aeration conditions in batch culture and under a variety of dilution rates in continuous culture under hypoxic conditions. The stoichiometric equivalence of 2-CBA degradation to chloride release under hypoxic conditions in the case of recombinant strains, and significant deviation from the same for the wild type cells was strongly evident. These differences are likely due to 2-CBA degradation by either the *ortho*-dehalogenation, modified *ortho*, unique *meta*-cleavage, or gentisate pathway by the recombinant strain and the *meta*-cleavage pathway leading to suicidal products by wild type cells. It is possible that the ability of the recombinant strain to maintain the ability to degrade 2-CBA by a non-suicidal pathway under hypoxic conditions is due to its ability to express VHb. This occurs presumably through VHb's enhancement of oxygen uptake by the cells, particularly under these conditions.

This research also extends the list of aromatics, the biodegradation of which can be enhanced by *vgb*/VHb under hypoxic conditions, to those containing chlorine. The plasmid bearing *vgb* was completely stable for one month in the recombinant strain grown in continuous culture. These results serve as a proof of concept to support future experiments to look for *vgb*/VHb related enhancement of 2-CBA degradation in contaminated soils, sediments, and water.

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