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## Effects of *Vitreoscilla* hemoglobin on the 2,4-dinitrotoluene (2,4-DNT) dioxygenase activity of *Burkholderia* and on 2,4-DNT degradation in two-phase bioreactors

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**Abstract** Expression of *vgb*, encoding *Vitreoscilla* hemoglobin (VHb), in *Burkholderia* strain YV1 was previously shown to improve cell growth and enhance 2,4-dinitrotoluene (2,4-DNT) degradation compared with control strain DNT, especially under hypoxic conditions. In the work reported here, the ratio of 2,4-DNT degraded to oxygen uptake was approximately 5-fold larger for strain YV1 than for strain DNT. The addition of purified VHb to cytosolic fractions of strain DNT increased 2,4-DNT degradation 1.5-fold, compared with 1.1-fold for control bovine Hb, but increased the 2,4-DNT degradation 2.7-fold when added to partially purified 2,4-DNT dioxygenase, compared with 1.3-fold for bovine Hb. This suggests a direct transfer of oxygen from VHb to the oxygenase. In a bioreactor at high 2,4-DNT concentration (using 100 ml oleyl alcohol containing 2 g 2,4-DNT as the second phase) with 1.5 l culture, both strains could remove 0.8 g 2,4-DNT by 120 h; and, under the same conditions in a fed-batch reactor, the degradation increased to 1 g for strain YV1 but not for strain DNT.

**Keywords** hemoglobin · 2,4-Dinitrotoluene · 2,4-DNT biodegradation · Two-phase bioreactors

### Introduction

Hemoglobin (VHb) from the gram-negative, gliding, filamentous, and strictly aerobic bacterium *Vitreoscilla* [33] is the best characterized prokaryotic Hb [40, 41]. Its function may be to enable the organism to survive in

oxygen-limited environments by facilitating oxygen diffusion to terminal oxidases under oxygen-limiting conditions [9, 20, 30, 34, 42]. Positive effects on growth and productivity due to engineering with VHb have been shown in bacteria [1, 3, 4, 10, 11, 21, 22, 25, 26, 39, 43, 44], fungi [7, 27], plants [15], and mammalian cells [32].

2,4-Dinitrotoluene (2,4-DNT) is an aromatic compound and the major impurity resulting from the manufacture of 2,4,6-trinitrotoluene (TNT). Waste from this process has contaminated waterways and soils and, because of its toxicity, 2,4-DNT is listed as a priority pollutant by the United States Environmental Protection Agency [19]. *Burkholderia* sp. strain DNT, formerly known as *Pseudomonas* sp. strain DNT, is an aerobic, gram-negative bacterium that can degrade 2,4-DNT as a source of carbon and energy under aerobic conditions. A biodegradation pathway for 2,4-DNT by *Burkholderia* sp. strain DNT was first proposed by Spanggard et al. [35]. 2,4-DNT dioxygenase is the first enzyme in this pathway and the genes encoding this enzyme were identified by Suen et al. [37]. Recent studies showed that VHb enhances the first step in 2,4-DNT degradation [12], that *Burkholderia* sp. strain DNT engineered with the gene (*vgb*) encoding VHb (strain YV1) enhances 2,4-DNT degradation [29, 31], and *vgb* is stably maintained and expressed in a functional form in strain YV1 [31]. Reports on the beneficial effects of VHb prompted this study to explore further the enhancement by VHb of 2,4-DNT biodegradation by *Burkholderia* sp. strain DNT in vivo and in vitro.

Regarding our in vivo experiments, bioremediation is an increasingly popular alternative to conventional physical and chemical methods for treating aromatic compounds. The use of a two-phase organic/aqueous bioreactor configuration has been shown to permit biological treatment of xenobiotic wastes, such as phenol [5] and a mixture of benzene, toluene, and *p*-xylene [6]. To test whether strain YV1 has advantages for 2,4-DNT degradation over strain DNT in bioremediation bioreactors, we examined their performance in one- and two-phase bioreactors.

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## Materials and methods

### Bacterial strains and cell growth

*Burkholderia* sp. strain DNT [35] was kindly supplied by Drs. J. Spain and S. Nishino of Tyndall Air Force Base (Fla.). *Burkholderia* sp. strain YV1 was constructed in our laboratory by transforming strain DNT with plasmid pSC160 [31]. Plasmid pSC160 was constructed by inserting *vgb*-bearing plasmid pUC8:16 into the broad-host-range vector pKT230 [25]; and *vgb* expression was under control of the native promoter in pSC160.

Cultures of *Burkholderia* strain DNT were maintained on agar plates containing 2,4-DNT minimal medium [35]. The cells were transferred from agar plates to sterilized tryptic soy broth (TSB) containing 30 g TSB l<sup>-1</sup> and grown in 500-ml flasks containing 250 ml medium for 18 h with shaking at 150 rpm at 37 °C. The cells were harvested by centrifugation at 6,000 g for 10 min and washed twice with 20 mM sodium phosphate buffer (pH 7.2). The washed cells were either stored at -80 °C until used for the purification of 2,4-DNT dioxygenase or resuspended in the same buffer to an appropriate cell concentration and kept on ice for the measurement of oxygen uptake and 2,4-DNT degradation rate.

*Burkholderia* strain YV1 was maintained and grown in the same way as *Burkholderia* strain DNT, except that 40 mg kanamycin (Kan) l<sup>-1</sup> and 100 mg ampicillin (Amp) l<sup>-1</sup> were added to agar plates and liquid media.

*Vitreoscilla* strain C1 was grown in PYA medium (1% peptone, 1% yeast extract, 0.02% sodium acetate, pH 7.8). The cells were harvested (as described above for *Burkholderia*) from 2,800-ml flasks containing 1,500 ml PYA after growth for 22 h with shaking at 200 rpm at 30 °C. The cells were washed twice with 0.01 M Tris buffer, pH 7.5, and kept on ice until used for the purification of Vhb.

### Cell lysis

Cells of *Burkholderia* strains were lysed by sonication. Freshly harvested cells were suspended in an equal volume (w/v) of deionized, sterilized water or 20 mM sodium phosphate buffer (pH 7.2) in a 15-ml plastic bottle and sonicated at 0 °C, using repeats of a 30 s burst with a 30 s interval and a 50% duty cycle at power level 7, using a Sonifier 350 (Branson, Danbury, Conn.), for a total of 1 min for each gram of cells. The lysis of *Vitreoscilla* was performed using the procedure of Georgiou and Webster [13].

### Partial purification of 2,4-DNT dioxygenase from *Burkholderia* strain DNT and purification of Vhb from *Vitreoscilla*

All purification procedures were carried out at 4 °C, unless stated otherwise. Cell extracts for enzyme purification were prepared from 15 g (wet weight) frozen cells suspended in an equal volume (w/v) of 20 mM sodium phosphate buffer (pH 7.2). The cell suspension was lysed as described above. Cytosolic fractions were prepared from the cell extracts by centrifugation at 45,000 g for 1 h and applied to a DEAE-cellulose column (2.5×40 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.2). The column was washed with about 1,000 ml of the same buffer. Bound proteins were eluted with a linear gradient of sodium chloride (0.0–0.5 M in the same buffer) at a flow rate of approximately 26 ml h<sup>-1</sup>. Fractions with 2,4-DNT degradation activity were pooled, concentrated using a concentrator (Amicon, Danvers, Mass.) or aquacide (Calbiochem, San Diego, Calif.), dialyzed in 20 mM sodium phosphate (pH 7.2), and chromatographed on a gel filtration column (Sephadex G150, 2.0×80 cm, flow rate 9.3 ml h<sup>-1</sup>) equilibrated with the same buffer. All fractions with 2,4-DNT degradation activity were collected and stored frozen at -20 °C. The purification of Vhb from *Vitreoscilla* was performed as described previously [8].

### Measurement of oxygen uptake and 2,4-DNT dioxygenase activity assay

Whole-cell oxygen uptake was measured with an oxygen monitor (model 53; Yellow Springs Instruments, Yellow Springs, Ohio) and a recorder (model SRG-2; Sargent-Welch, Skokie, Ill.). The temperature was controlled at 37 °C by a circulator (model 1267-62; Cole Parmer, Chicago, Ill.). The cells were grown and harvested as described above. The 2,4-DNT solution was adjusted to an appropriate concentration with water. The cells and 3 ml air-saturated 2,4-DNT solution were loaded into the respirometer chamber. When the oxygen concentration decreased to 50%, the cell suspension was removed from the chamber and centrifuged immediately. The supernatant was saved and the 2,4-DNT disappearance was calculated by measuring the absorbance at 254 nm ( $A_{254}$ ).

For cell extracts and purified fractions, 0.02 ml were loaded into the respirometer chamber with air-saturated 2,4-DNT substrate solution (2 ml of 5.5×10<sup>-4</sup> M 2,4-DNT, 0.1 ml of 10 mM NADH in 20 mM sodium phosphate buffer, pH 7.2) at 37 °C. After 3 min, aliquots were removed from the chamber to measure 2,4-DNT disappearance by high-pressure liquid chromatography (HPLC). Alternatively, previously frozen fractions were assayed in 1.5-ml microtubes containing 0.5 ml air-saturated 5.5×10<sup>-4</sup> M 2,4-DNT and 1.0 mM NADH in 20 mM sodium phosphate buffer, pH 7.2. The microtubes were equilibrated in a 37 °C water bath for 5 min and then 20 μl of the thawed samples were added and the tubes were incubated for another 5 min. The 2,4-DNT disappearance was measured by HPLC.

### Measurement of 2,4-DNT concentration using HPLC and $A_{254}$

The HPLC system used was from Varian (Walnut Creek, Calif.), using solvent delivery system 9012 Q and variable wavelength UV-VIS detector 9050. After a minimum 5 min warm-up, 50 ml of combined solvent [1:1 v/v 0.1% trifluoroacetic acid (v/v in water) with acetonitrile (HPLC grade)] were used to purge the solvent line. Then, the combined solvent was pumped (1.4 ml min<sup>-1</sup> with constant pressure) through a C18 column (150×4.6 mm; Varian) and 5 μl of sample were loaded with a 10-μl syringe into the injector (Rheodyne, Cotati, Calif.). All data were collected by a computer with the Star Chromatography Workstation software (Varian). For the measurement of 2,4-DNT in oleyl alcohol, the HPLC conditions were the same as those above (2,4-DNT in aqueous solvents), except the 2,4-DNT samples were diluted 1:100 (1:10 serial dilution twice) with absolute ethanol, the combined solvent was 1:1 ethanol (HPLC grade) and acetonitrile, and the C18 column was 250×4.6 mm (Resolution Systems, Holland, Mich.). The 2,4-DNT extinction coefficient (15.2 mM<sup>-1</sup> cm<sup>-1</sup>) was determined at 254 nm.

### Kinetic measurements for 2,4-DNT dioxygenase

In a microtube, 10 μl of 20 mM NADH in 20 mM sodium phosphate buffer (pH 7.2) were added to a 10 μl enzyme sample and incubated in a 37 °C water bath for 1 min. Then, 20 μl (37 °C) of varying concentrations of 2,4-DNT in 20 mM sodium phosphate buffer (pH 7.2) were added to the tube. This tube was flicked, centrifuged to sediment any droplets, and returned to the 37 °C water bath for 10 s. The 2,4-DNT disappearance from the sample was immediately measured by HPLC. Kinetic parameters ( $K_{M,2,4-DNT}$ ,  $V_{max,2,4-DNT}$ ) were determined from Lineweaver-Burk plots.

### Measurements of protein concentrations and SDS-PAGE

Protein concentrations in cell extracts and column fractions were determined by the Folin-Ciocalteu method, as described by Switzer and Garrity [38], using the  $A_{280}$  values with lysozyme as

the standard. Protein concentrations in the cytosolic fractions (centrifuged cell extracts) of *Burkholderia* sp. strain DNT were determined by the dye-binding assay [2], using bovine serum albumin as the standard. For SDS-PAGE, 15% polyacrylamide gels were prepared as described by Laemmli [24] and used to analyze the 2,4-DNT dioxygenase fractions from the G150 Sephadex chromatography. The gels were stained by the silver-staining method [28].

Determination of the native molecular weight of 2,4-DNT dioxygenase by gel filtration on Bio-Gel P-300

Standard proteins [cytochrome *c* (13 kDa), myoglobin (16.9 kDa), egg albumin (45 kDa), Hb (64 kDa),  $\beta$ -galactosidase (116 kDa), myosin (200 kDa)] were individually chromatographed to determine their elution times. Their elution volume ( $V$ ) was calculated by multiplying the flow rate (10 ml  $h^{-1}$ ) by the peak elution time. Blue dextran (2,000 kDa) was used to measure  $V_0$ , the void volume (22 ml). The standard curve was constructed by plotting  $V/V_0$  versus log molecular mass.

Effects of VHb and bovine Hb on 2,4-DNT degradation by cell extracts of *Burkholderia* sp. strain DNT and purified 2,4-DNT dioxygenase

Cytosolic fractions of *Burkholderia* were obtained by centrifuging lysed cells at 10,000  $g$  for 10 min at 4 °C. In microtubes, 50  $\mu$ l of 200 mM NADH in 20 mM sodium phosphate buffer, pH 7.2, were added to 50  $\mu$ l of 40  $\mu$ M Hb. The tubes were incubated at room temperature for at least 10 min. Then, in order, 50  $\mu$ l of 0.1 mM 2,4-DNT in the same phosphate buffer and 50  $\mu$ l of lysed cells, cytosolic fractions, or purified 2,4-DNT dioxygenase were added to the tubes. The tubes were incubated in a 37 °C water bath for 10 min (20 min for the purified enzymes). The 2,4-DNT disappearance from these samples was measured by HPLC.

One- and two-phase bioreactors for 2,4-DNT bioremediation

A 2-l fermentor (model F-2000, New Brunswick Scientific Co., Edison, N.J.) was used in all experiments. The conditions common to the five types of experiment performed were as follows. The basic medium (1.5 l) was either 2,4-DNT minimal medium [35] or TSB, which was saturated with oxygen by using forced air before inoculation. The inoculum was 4 ml of a TSB overnight culture. During growth, temperature was controlled at 37 °C, the culture was always agitated, and the pH was monitored continuously. According to the parameter(s) being investigated, individual experiments varied in the following ways: (1) additions of oleyl alcohol, glucose, or 2,4-DNT were made (or not) to the basic medium, (2) the strain used did (or did not) contain *vgb*, (3) pH was (or was not) controlled, (4) forced air was (or was not) added during the run, (5) the number of impellers and the impeller speed was varied, and (6) the mode used was batch or fed-batch.

For the initial investigation of the abilities of strains DNT and YV1 to degrade 2,4-DNT in bioreactors, 2,4-DNT minimal medium plus 5 g glucose  $l^{-1}$  was used, there was one impeller at 400 rpm, and there was no pH control. For high aeration, forced air was added at a rate of 0.04  $m^3 h^{-1}$  and, for hypoxic conditions, no forced air was added. For all other runs, forced air was added at a rate of 0.04  $m^3 h^{-1}$ . For measuring the effects of oleyl alcohol on growth, TSB was used with or without the addition of 100 ml of oleyl alcohol, one impeller at 400 rpm was used, and there was no pH control. Two sets of conditions (A, B; Table 2) were used to investigate 2,4-DNT degradation in batch mode with the two-phase system. The fed-batch conditions were the same as condition B in Table 2, except for the addition of 10 ml of 10 $\times$  TSB at 24-h intervals.

## Results

### Partial purification of 2,4-DNT dioxygenase

Table 1 shows that the total purification of 2,4-DNT dioxygenase from the cytosol of *Burkholderia* sp. strain DNT was 20.3-fold. The purest fraction from Sephadex G150 chromatography was analyzed on a SDS-PAGE gel. It was estimated to be about 5–10% pure by visual examination and four bands at the molecular sizes expected for the subunits [37] were identified. The native molecular mass of 2,4-DNT dioxygenase determined by gel filtration was 187 kDa; and the molecular size calculated from the deduced amino acid sequences of its subunits, including one ORF of unknown function, was 171 kDa [37]. The  $K_M$  for 2,4-DNT dioxygenase, determined using the partially purified enzyme, was 40  $\mu$ M, in good agreement with the 27  $\mu$ M obtained using cell extracts [12].

### 2,4-DNT biodegradation relative to oxygen consumption for whole cells of *Burkholderia* sp. strains DNT and YV1

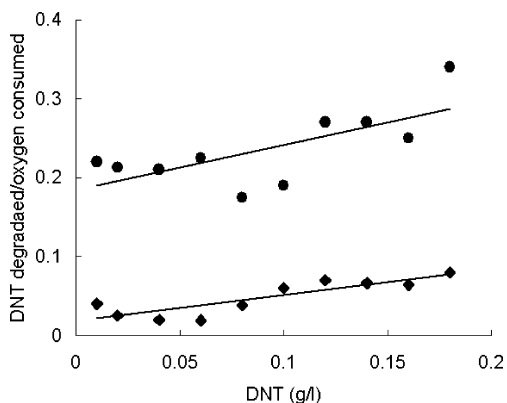
The increased efficiency of 2,4-DNT degradation by cells of strain YV1 (that contain VHb) is shown in Fig. 1, which relates the ratio of 2,4-DNT degraded to oxygen uptake at varying 2,4-DNT concentrations. This ratio was roughly 5-fold higher for strain YV1 than for strain DNT at all concentrations of 2,4-DNT.

### Effects of Hb on 2,4-DNT degradation by lysed cells and cytosolic fractions of *Burkholderia* sp. strain DNT and by purified 2,4-DNT dioxygenase

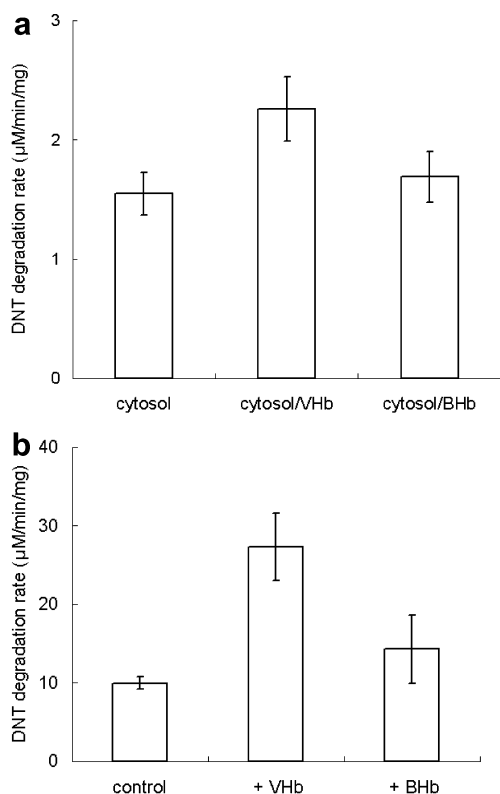
VHb increased 2,4-DNT degradation 46% by the cytosol, compared with a 9% stimulation by bovine Hb (BHb; Fig. 2a), but increased it 174% for purified 2,4-DNT dioxygenase, compared with only 32% for BHb (Fig. 2b).

**Table 1** Purification of 2,4-dinitrotoluene (DNT) dioxygenase from 15 g (wet weight) of cells of *Burkholderia* sp. strain DNT. Protein content was determined as described in Materials and methods. Specific activity was determined in a respirometer chamber, as described in Materials and methods. 2,4-DNT concentration was measured by HPLC

Purification step	Protein (mg)	Specific activity ( $\mu$ M $min^{-1}$ $mg^{-1}$ )	Recovery (%)	Purification ( $\times$ -Fold)
Cytosol	539.0	19	100.0	1.0
DEAE-Cellulose	32.9	206	30.4	10.8
Sephadex G150	9.32	385	12.4	20.3



**Fig. 1** Ratio of 2,4-dinitrotoluene (2,4-DNT) degradation to oxygen uptake for whole cells at different 2,4-DNT concentrations. This ratio was roughly 5-fold higher for strain YV1 than for strain DNT at all concentrations of 2,4-DNT. 2,4-DNT concentrations were calculated from measuring the absorbance at 254 nm ( $A_{254}$ ). Circles Strain YV1, diamonds strain DNT



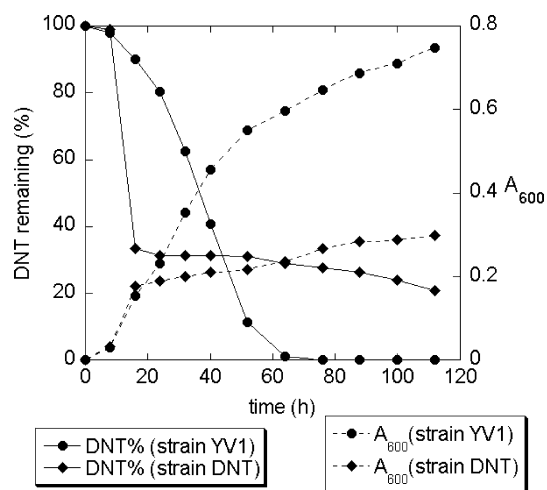
**Fig. 2a, b** Effect of *Vitreoscilla* (VHb) and bovine (BHb) hemoglobins. **a** Effect of hemoglobins on 2,4-DNT degradation by cytosolic fractions of *Burkholderia* sp. strain DNT. **b** Effects of hemoglobins on partially purified 2,4-DNT dioxygenase activity. The G150 Sephadex fraction containing the highest enzyme activity was used for these experiments. For both **a** and **b**, 2,4-DNT dioxygenase activity was determined in 1.5-ml microtubes, as described in Materials and Methods; and 2,4-DNT concentration was measured by HPLC. Values are averages of five individual measurements (standard deviations are indicated)

## 2,4-DNT degradation in bioreactors by *Burkholderia* sp. strains DNT and YV1

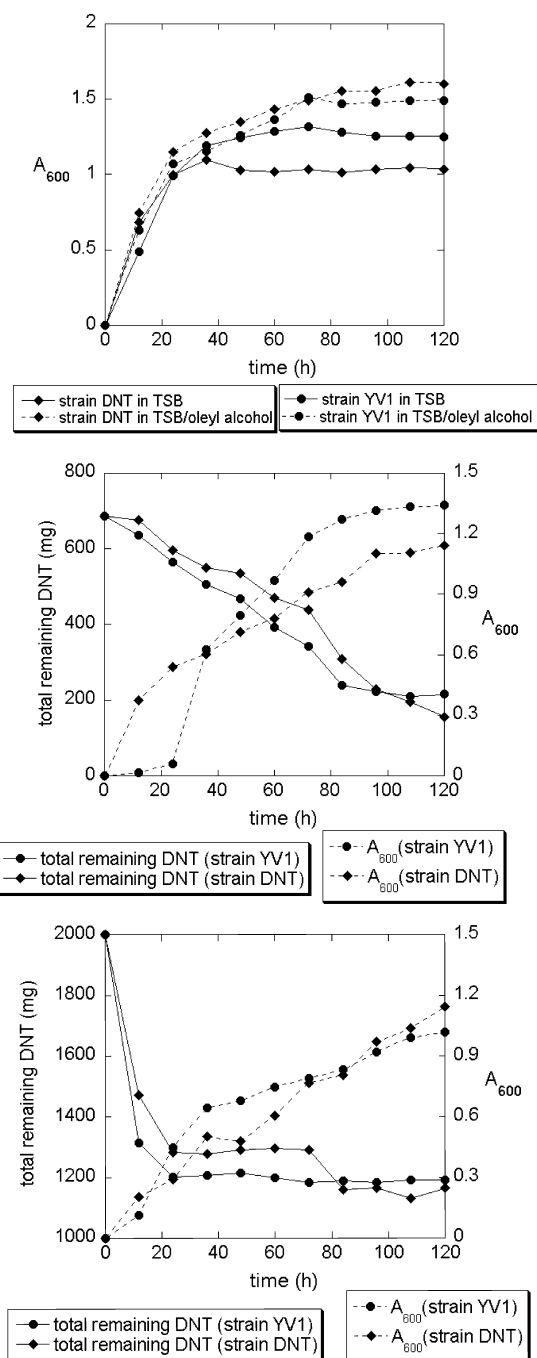
Glucose ( $5 \text{ g l}^{-1}$ ) was selected as a carbon source for both strains to enhance 2,4-DNT degradation by co-metabolism. Under high aeration, strain YV1 showed no advantage in growth or in 2,4-DNT degradation over the control strain DNT; and this was observed previously [12, 29]. Under hypoxic conditions, however, strain YV1 did show advantages over strain DNT (Fig. 3). Although strain DNT initially consumed 2,4-DNT at a faster rate in this one-phase bioreactor experiment, both its growth and 2,4-DNT degradation leveled off, whereas strain YV1 consumed 100% of the 2,4-DNT and grew to a cell density about 3-fold larger.

One hindrance to the bioremediation of 2,4-DNT is its low solubility in water,  $187 \text{ mg l}^{-1}$  [35]. The solubility of 2,4-DNT in oleyl alcohol was tested and found to be about  $20 \text{ g l}^{-1}$ , more than 100-fold greater than its solubility in water. But 2,4-DNT also showed high diffusibility from oleyl alcohol to water, the concentration in the aqueous phase reaching  $230 \text{ mg l}^{-1}$  after 2 h. This is a value 23% higher than the solubility,  $187 \text{ mg l}^{-1}$ , reported by Spanggard et al. [35]. This increased solubility is likely due to the different conditions we used, i.e., temperature and agitation. The growth of *Burkholderia* in a two-phase system compared with a one-phase system using TSB as the medium is shown in Fig. 4 (top panel) for strains DNT and YV1. Surprisingly, both strains grew better in the two-phase bioreactor, i.e., in the presence of oleyl alcohol; and, because of this result, oleyl alcohol was selected to be the 2,4-DNT carrier to enhance 2,4-DNT degradation in two-phase bioreactors.

Table 2 shows the two set-up conditions (A, B) for the two-phase bioreactors. In condition A, after an ini-



**Fig. 3** 2,4-DNT degradation by and growth of *Burkholderia* sp. strains DNT and YV1 in bioreactors with 2,4-DNT minimal medium and glucose (no aeration). 2,4-DNT disappearance was measured by HPLC. Each sample taken from the medium was 1 ml. The first six samples were taken at 8-h intervals and the following samples were taken at 12-h intervals



**Fig. 4** Growth of *Burkholderia* sp. strains DNT and YV1. *Top panel* Growth of strains DNT and YV1 in tryptic soy broth (TSB) medium with and without oleyl alcohol in the bioreactor. Parameters were the same as condition A in Table 2, except there was no 2,4-DNT in the oleyl alcohol. *Solid lines* represent the one-phase bioreactors with 1.5 l of TSB medium and *dashed lines* represent the two-phase bioreactors with 1.5 l of TSB medium plus 100 ml oleyl alcohol. *Middle panel* 2,4-DNT degradation by and growth of *Burkholderia* sp. strains DNT and YV1 in the two-phase bioreactor under condition A of Table 2. The total remaining 2,4-DNT was measured by HPLC and calculated by combining the remaining 2,4-DNT in the aqueous phase with the remaining 2,4-DNT in the oleyl alcohol phase. *Bottom panel* 2,4-DNT degradation by and growth of *Burkholderia* sp. strains DNT and YV1 in the two-phase bioreactor under condition B of Table 2. The total remaining 2,4-DNT was measured and calculated as in the middle panel

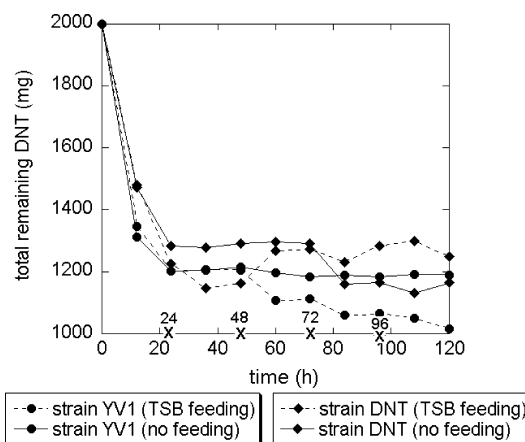
**Table 2** Two conditions used for two-phase bioreactors for 2,4-DNT bioremediation. The initial inoculum was 4 ml for both. The temperature was set at 37 °C. The initial volume of the tryptic soy broth medium was 1.5 l and the aeration rate was 0.04 m<sup>3</sup> h<sup>-1</sup> for both conditions

Parameter	Conditions	
	A	B
Oleyl alcohol	50 ml with 700 mg 2,4-DNT	100 ml with 2,000 mg 2,4-DNT
pH control	None	Feedback between 7.0 and 7.5
Impeller	One	Two
Agitation	400 rpm	300 rpm

tial lag phase, strain YV1 had a faster growth rate and had the higher 2,4-DNT degradation rate until after 100 h (Fig. 4, central panel). In condition B, there was more 2,4-DNT and one more impeller was added for better mixing of the two phases. This resulted in a steep initial 2,4-DNT degradation rate for about the first 24 h for both strains, which then leveled off for the rest of the experiment (Fig. 4, bottom panel). There was little difference in growth rate and 2,4-DNT degradation for the two strains under these conditions. In a fed-batch experiment, aliquots of 10 ml of 10× TSB medium were added to the two-phase bioreactor under condition B every 24 h after the first 24 h. Strain YV1 but not strain DNT showed a better 2,4-DNT degradation rate after 48 h under these conditions, compared with the non-fed conditions, degrading half (about 1 g) of the total 2,4-DNT in the reactor (Fig. 5).

## Discussion

In the one-phase bioreactor (Fig. 3), the beneficial effects of Vhb occurred only under hypoxic conditions; and similar results were previously observed in 2,4-DNT



**Fig. 5** 2,4-DNT degradation by *Burkholderia* sp. strains DNT and YV1 in a two-phase fed-batch bioreactor under condition B of Table 2. *The crosses* on the abscissa indicate the times when 10× TSB medium was added. The total remaining 2,4-DNT was measured and calculated as in Fig. 4, middle panel

degradation [12, 29] and in other systems [3, 17, 20]. Two major systems involved in oxygen consumption by strain DNT are the respiratory chain and the oxygenases in the 2,4-DNT degradation pathway. Thus, stimulation of 2,4-DNT degradation by VHb observed in vivo under hypoxic conditions could be a direct effect, resulting from increased oxygen delivery to the oxygenases, or an indirect effect on cell respiration. The latter could be an increased respiratory efficiency at low oxygen levels leading to higher ATP synthesis and increased protein (i.e., oxygenase) synthesis; and there is evidence for this in other systems [16, 17, 22]. Previous work showed that cells of strains expressing *vgb* did have a higher level of 2,4-DNT dioxygenase activity [12] and the increased efficiency of 2,4-DNT degraded per oxygen consumed observed here (Fig. 1) supports this. However, this result and the direct stimulation of 2,4-DNT degradation by exogenous VHb for both cytosolic fractions and partially purified 2,4-DNT dioxygenase (Fig. 2) are evidence that VHb directly stimulates this enzyme, presumably by delivering oxygen to it. These results also show the superiority of VHb relative to BHb for this purpose; and this has been observed for other systems [18]. The purest fractions containing 2,4-DNT dioxygenase were not likely to contain DntB and DntC, because the three oxygenases have molecular sizes of 171 kDa, 60 kDa, and 44 kDa, respectively [14, 36], which would result in their separation in the gel filtration chromatography step. Thus, the effects of Hbs on 2,4-DNT degradation by the purified 2,4-DNT dioxygenases are likely due to interactions solely with that enzyme. It appears, then, that 2,4-DNT dioxygenase activity is elevated both by increased production and by a direct effect of VHb on the enzyme.

Neither strain could completely degrade the relatively large amount of 2,4-DNT in the two-phase bioreactors under the conditions tested, but under condition B, 1.5 l of culture was capable of degrading gram quantities of 2,4-DNT (Figs. 4, 5). Leveling-off of the degradation at the end of the experiment was not due to nutrient deficiency, since adding additional nutrient at these times did not result in further degradation. The amount of 2,4-DNT degraded was approximately 0.8 g for both strains in the two-phase bioreactor under condition B. To degrade this amount would require about 5 l of a TSB culture in a one-phase bioreactor, a saving of 3.5 l of TSB medium for the two-phase partitioning bioreactor. The two-phase bioreactor has another advantage. The 2,4-DNT in a contaminated site could be extracted with an organic solvent, the 2,4-DNT transferred from this primary solvent to oleyl alcohol, and degraded in a two-phase bioreactor. Both the extraction solvent and the oleyl alcohol would then be available for further cycles of extraction and biodegradation.

Surprisingly, strains YV1 and DNT grew better in the presence of oleyl alcohol in the two-phase bioreactor than in its absence (Fig. 4, top panel). There is evidence that *Pseudomonas putida* S12 has an efflux pump involved in solvent tolerance [23], but whether *Burk-*

*holderia* has any such efflux pump is not known. The possibility that *Burkholderia* may be able to use oleyl alcohol as a carbon source also needs to be examined.

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