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Vitreoscilla hemoglobin enhances the first step in 2,4-dinitrotoluene degradation in vitro and at low aeration in vivo

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Abstract

The initial enzyme in 2,4-dinitrotoluene (2,4-DNT) catabolism from *Burkholderia* sp. strain DNT is DNT dioxygenase, which catalyzes oxygen addition to 2,4-DNT to form 4-methyl-5-nitrocatechol (MNC). The gene for this enzyme, *dntA*, was placed into *Escherichia coli* both in the presence and absence of the *Vitreoscilla* hemoglobin gene, *vgb*, producing strains PF6 and PFJS39, respectively. PF6 outgrew PFJS39 in LB medium and at restricted aeration in minimal medium containing 110 μM (20 ppm) 2,4-DNT. When grown in minimal medium containing 110 μM 2,4-DNT with normal aeration, the two strains converted 2,4-DNT to MNC at almost the same rate, while with restricted aeration the rate for PF6 was twice that of PFJS39. The V_{max} and K_M for 2,4-DNT for the conversion was determined using whole cells and whole cell lysates of both strains. For cells grown under both normal and restricted aeration in minimal/110 μM 2,4-DNT medium there was a three- to four-fold increase in V_{max} for PF6 compared to PFJS39 but also about a 50% increase in the apparent K_M. These results support the idea that *Vitreoscilla* hemoglobin can enhance bioremediation pathways of aromatic compounds that require oxygen addition at one or more steps.

Keywords: *Vitreoscilla* hemoglobin; Bacterial hemoglobin; 2,4-Dinitrotoluene degradation

1. Introduction

The bacteria *Vitreoscilla* are strict aerobes but have a tendency to grow in oxygen-poor environments [1]. They have adapted a mechanism, novel for bacteria, to grow under these conditions: they synthesize a bacterial hemoglobin (VHb) in response to hypoxic conditions [2,3]. Until the discovery of VHb, it was generally believed that hemoglobins evolved in higher organisms and were lacking in bacteria [4]. The putative function of VHb is to trap extracellular oxygen and feed it to the membrane terminal oxidases; the kinetic constants for oxygen binding to VHb reflect this function. VHb has a somewhat larger rate constant for oxygen association (k_{on}) than horse myoglobin, but its rate constant for oxygen dissociation (k_{off}) is over 500 times larger (which accounts for its larger K_d). The larger k_{on} reflects a higher ''avidity''

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for oxygen, but its much larger k_{off} enables it to release the bound oxygen more readily than Mb and almost all other hemoglobins [2].

Another observation that has been reported relevant to the significance of the role of VHb is that its expression (via genetic engineering with the VHb gene, *vgb* [5,6]) often improves growth as well as protein and metabolite synthesis in bacteria and fungi [7–16]. In particular, we showed that this enhancement can be extended to members of the *Pseudomonadaceae*, an obligately aerobic family of bacteria which are able to biodegrade a wide variety of toxic chemicals [14]. We also showed that under certain conditions, biodegradation of benzoic acid can be enhanced by genetic engineering of *Xanthomonas maltophilia* to produce VHb [17]. A working hypothesis is that VHb enhances oxygen uptake both to produce more ATP for cell growth by oxidative phosphorylation and to degrade aromatic compounds, pathways for which generally depend on oxygen addition to the aromatic ring [18].

In this regard, application of *vgb*/VHb technology to in situ bioremediation of a variety of aromatic contaminants from soil and groundwater may be useful. The possibility exists that the presence of VHb in engineered microorganisms will result in enhanced growth and aromatic compound degradation near the surface where oxygen is not limiting, but will afford particular advantages in these two capabilities below the surface, where oxygen is limiting. As a model system for this approach, we have used degradation of 2,4-dinitrotoluene (2,4-DNT) by *Burkholderia* sp. strain DNT [19,20]. Our initial studies indicate that the presence of *vgb*/VHb does enhance this process [21,22]. In the work presented here, we made use of the cloned gene for the first enzyme in this pathway, DNT dioxygenase [20,23], to examine some of the biochemical details of this enhancement. In particular, we investigated whether *vgb*/VHb results in a stimulation of the first step in 2,4-DNT metabolism, during which oxygen is first utilized.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

Escherichia coli was strain JM109 [24]. Plasmids were pJS39 (a gift of Dr. J. Spain [20]) and pHG1 (produced in our laboratory by H. Geckil). Plasmid pJS39 bears the DNT dioxygenase genes (*dntAa–dntAd* [23]) from *Burkholderia* sp. strain DNT in Promega vector pGEM7Z(+) [20]; pHG1 contains the *Vitreoscilla* hemoglobin gene, *vgb*, in vector pVK102 [25,26]. Cells were transformed with pHG1 (strain PFHG1), pJS39 (strain PFJS39), or both plasmids (strain PF6) using the $CaCl_2$ method [27]. Strains were maintained on Luria–Bertaini (LB) plates containing 100 μg/ml ampicillin (PFJS39), 12.5 μg/ml tetracycline (PFHG1) or both antibiotics at these levels (PF6).

For growth in LB medium inocula of PFHG1 and PF6 were prepared in LB supplemented with the appropriate antibiotics (at the levels described in the preceding paragraph). After culturing at 150 rpm and 37°C for 16 h, culture samples were collected by centrifugation, washed twice with and resuspended in fresh LB (containing the appropriate antibiotics as described above). An equal number of A_{600} units of each strain was then inoculated into 200 ml of fresh LB (again containing the appropriate antibiotics at the levels described above) in 1000 ml Erlenmeyer flasks and incubated at 150 rpm and 37°C.

DNT minimal medium (for growth of PFJS39 and PF6) was that previously reported by Spanggord et al. [19], but containing the following additional components: 2.36 g/l succinic acid (stock solutions adjusted to pH 7.2 before addition), 5 μg/ml proline, 0.6 μg/ml thiamine–HCl, and, for the sake of consistency, 100 μg/ml ampicillin for both strains (pHG1 was found to be completely stable for at least 24 h in culture in the absence of tetracycline). This medium also contained 110 μM (20 ppm) 2,4-DNT. In some experiments, the succinic acid was replaced with 10 g/l glucose; this was

done to reduce hemoglobin levels, as *vgb* expression is subject to catabolite repression [3]. Inocula were prepared and cultures inoculated as described in the previous paragraph except that this minimal medium was used. For the actual experiments normal aeration conditions were 150 rpm, 37°C with 200 ml of medium in 1000 ml Erlenmeyer flasks, while restricted aeration conditions were 50 rpm, 37°C with 200 ml of medium in 250 ml flasks.

2.2. In vitro experiments with strains PFJS39 and PF6

To prepare whole cells or whole cell lysates of strains PFJS39 or PF6, cells were grown in 200 ml minimal medium containing 110 μM 2,4-DNT, 100 μg/ml ampicillin, and 2.36 g/l succinic acid using either the normal or restricted aeration conditions described in the previous paragraph. Cultures were grown for 18 h to ensure production of DNT dioxygenase. Cells were harvested by centrifugation, washed and resuspended in 0.05 M sodium phosphate, pH 7.0 (also containing 2,4-DNT at concentrations varying from 10 to 150 μM), to a final concentration of 1.5 A_{600} units. Such cells were used directly or converted to whole cell lysates by treatment with 10 μg/ml lysozyme for 10 min at room temperature.

2.3. Analytical methods

Plasmid mini-preparations were performed as described by Holmes and Quigley [28] and analyzed on 1% agarose gels in 0.04 M Tris–acetate, 1 mM EDTA, pH 7.0 containing 10 μg/ml ethidium bromide. Hemoglobin concentrations were determined by whole cell CO-difference spectra as described previously [5,29]. Production of 4-methyl-5-nitrocatechol (MNC) was monitored by increase in absorbance at 417 nm (the absorbance maximum at both pH 7.0 and 8.0 in the visible range determined from spectral analysis of purified MNC, a gift of Dr. J. Spain; we measured an extinction coefficient of 0.0055 $\mu M^{-1}\ cm^{-1}$ for MNC at 417 nm and pH 7.0). Culture samples, or samples of whole cells or cell lysates were clarified by centrifugation before spectrophotometry; A_{417} values of all samples were on the linear portion of an A_{417} vs. MNC concentration standard curve. For DNT dioxygenase specific activity determinations, cell samples were lysed with lysozyme and protein determined using the Lowry method [30]. Cell growth was monitored both by absorbance at 600 nm (with culture samples diluted as necessary with fresh medium so as to keep the measured absorbances below 0.6) and by plating on LB plates without antibiotics after serial dilution in 0.85% NaCl. Following colony growth and enumeration 50 colonies were transferred to plates containing appropriate antibiotics to check for plasmid stability, which was at least 96% for both plasmids for all of the data reported here.

3. Results

3.1. Construction and confirmation of strains

Plasmid pJS39 was successfully transformed into *E. coli* JM109 to form strain PFJS39. PFJS39 was then transformed with the *vgb*-bearing plasmid pHG1 to form strain PF6. A third control strain (PFHG1) was constructed by transforming *E. coli* JM109 with pHG1. The presence of the various plasmids in each strain was confirmed by agarose gel electrophoresis (Fig. 1). It was also confirmed that both PF6 and PFHG1 produce VHb (Table 1); PF6 generally produced about 50% more VHb than PFHG1, but both strains produced roughly the same amount of VHb as our other recombinant *E. coli* strains bearing *vgb* [3]. Thus, the presence of pJS39 and the dioxygenase gene does not negatively affect VHb production. For both strains VHb levels increased in late log phase (12 h) and decreased in stationary phase (15–24 h).

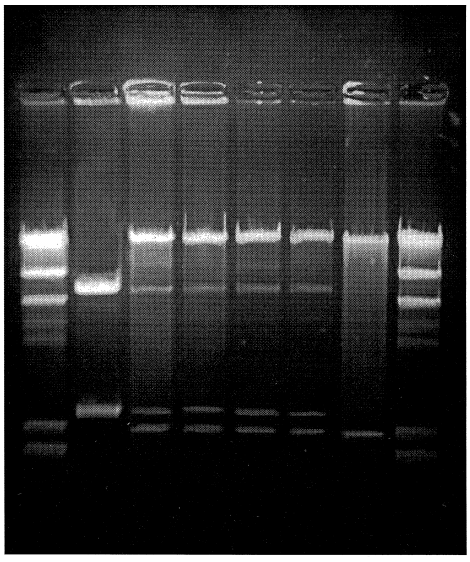

Fig. 1. Agarose gel electrophoretic analysis of plasmids from PFJS39, PFHG1, and PF6 cleaved with *Hin*dIII. Lanes 1 and 8, lambda phage DNA cleaved with *Hin*dIII; lane 2, PFJS39 (the expected bands at 6.8 and 2.8 kb of pJS39 are evident); lane 7, PFHG1 (the expected bands at 23 and 2.3 kb of pHG1 are evident); lanes 3–6, PF6 (the four bands expected for both pJS39 and pHG1 are evident).

3.2. Growth of and 2,4-DNT degradation by PF6 and PFJS39

In LB medium, growth (as measured by A_{600}) was inhibited about 25% by the presence of pJS39, and thus *dntA* (comparison of strains PFHG1 and PF6); the presence of *vgb* afforded about a 15%–20% growth advantage for *dntA*-bearing strains (comparison of PF6 and PFJS39). On a viable cell basis, both PF6 and PFHG1 outgrew PFJS39 by about two-fold throughout most of the growth curve.

Growth and 2,4-DNT conversion to MNC in minimal medium containing 110 μM DNT, 2.36 g/l succinic acid, and 100 μg/ml ampicillin was compared in shake flasks under two conditions, normal and restricted aeration, as described in Section 2. Under normal aeration

Table 1
Summary of hemoglobin levels in strains PF6 and PFHG1 as a function of time in culture. Growth in LB medium and determination of VHb were as described in Section 2. All values are averages of four independent trials, each being the average of three individual measurements (standard deviations in parentheses)

Time (h)	VHb content (nmol/g wet weight)
PF6	
9	158 (13)
12	395 (56)
15	347 (20)
24	166 (17)
PFHG1	
9	155 (31)
12	234 (62)
15	124 (33)
24	109 (36)

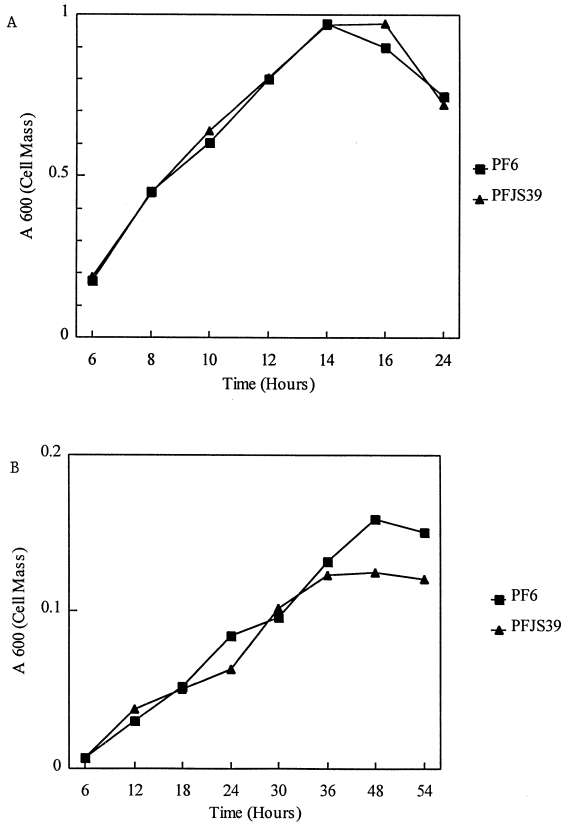

Fig. 2. Growth (A_{600}) of strains PF6 and PFJS39 in minimal medium with 110 μM (20 ppm) 2,4-DNT, 2.36 g/l succinic acid, and 100 μg/ml ampicillin. (A) normal aeration and (B) restricted aeration. Data points are the averages of nine individual experiments. The coefficients of variation for these points (calculated from σ_{n-1}) ranged from 2%–33% for PF6 and 1%–20% for PFJS39 in (A); and from 24%–27% for PF6 and 24%–29% for PFJS39 in (B).

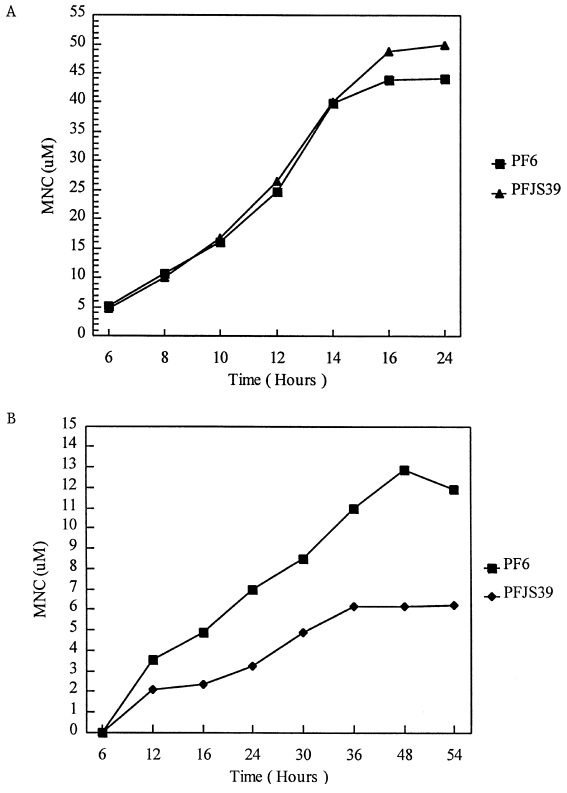

Fig. 3. MNC production as a function of time in culture for strains PF6 and PFJS39 for the growth experiments shown in Fig. 2. (A) Normal aeration and (B) restricted aeration. Data points are the averages of nine individual experiments. The coefficients of variation for these points (calculated from σ_{n-1}) ranged from 2%–36% for PF6 and 4%–20% for PFJS39 in (A); and from 7%–9% for PF6 and 4%–11% for PFJS39 in (B).

growth of the two strains as monitored by A_{600} was almost identical (Fig. 2A), although PF6 viable counts averaged about 50% of those for PFJS39 throughout the growth curve. At restricted aeration, the growth of both strains was about six to eight times lower on an A_{600} basis than at high aeration, and PF6 eventually (in late log phase) outgrew PFJS39 by about 25% (Fig. 2B). At restricted aeration, the numbers of viable cells of the two strains were very similar to each other throughout the growth curve and 20–40 times lower (at maximum density) than at high aeration. Plasmid stability for both strains/plasmids was 98% or greater at all times under both aeration conditions.

In the minimal-DNT-succinate medium under normal aeration, conversion of 2,4-DNT to MNC was similar for both strains; by stationary phase approximately half of the 2,4-DNT originally in the medium had been converted (Fig. 3A). In the same medium under restricted aeration conditions the conversion of 2,4-DNT to MNC was much lower for both strains (as expected because of the much lower growth), but PF6 converted more than twice as much 2,4-DNT to MNC as PFJS39 (Fig. 3B).

The substitution of 10 g/l glucose for 2.36 g/l succinic acid in minimal-DNT medium under conditions of restricted aeration resulted in a modest decrease (about 25%–35%) in growth (on an A_{600} basis) of both PF6 and PFJS39, but a fairly large increase in conversion of 2,4-DNT to MNC (Fig. 4). The relative advantage of PF6 over PFJS39 regarding 2,4-DNT catabolism, however, was reduced by about half compared to growth with succinate. With glucose and restricted aeration, the viable cell counts of the two strains were almost identical at all times, although glucose resulted in much greater viability than succinate at culture times greater

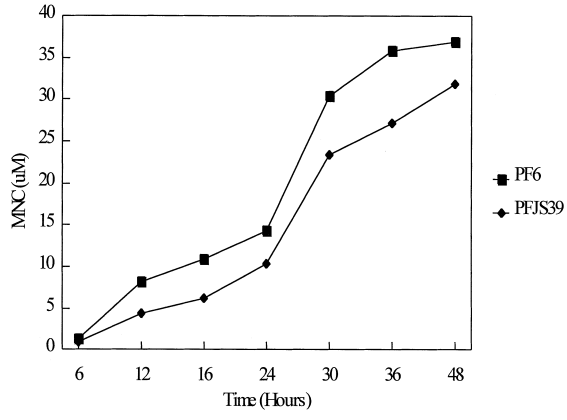

Fig. 4. MNC production as a function of time for strains PF6 and PFJS39 grown in minimal medium with 110 μM (20 ppm) 2,4-DNT, 10 g/l glucose, and 100 μg/ml ampicillin with restricted aeration. Data points are the averages of nine individual experiments. The coefficients of variation for these points (calculated from σ_{n-1}) ranged from 1%–11% for PF6 and 0.2%–16% for PFJS39.

than 36 h; this might account for the greater 2,4-DNT metabolism with glucose.

Growth of PF6 and PFJS39 with normal aeration was also compared in minimal medium containing 2.36 g/l succinic acid, 100 μg/ml ampicillin, and 25 or 50 μM MNC. With both MNC concentrations PF6 outgrew PFJS39 (A_{600} basis) by two-fold. The basis for this *vgb*/VHb-related increase in resistance to MNC toxicity is not known.

3.3. In vitro experiments

The K_M and V_{max} for 2,4-DNT of DNT dioxygenase were determined in whole cells and whole cell lysates of PFJS39 and PF6 grown at both normal and restricted aeration (as described in Section 3.2). Conversion of 2,4-DNT to MNC was approximately linear for 14 h at 2,4-DNT concentrations of 10 to 150 μM in all cases. When these data were converted to

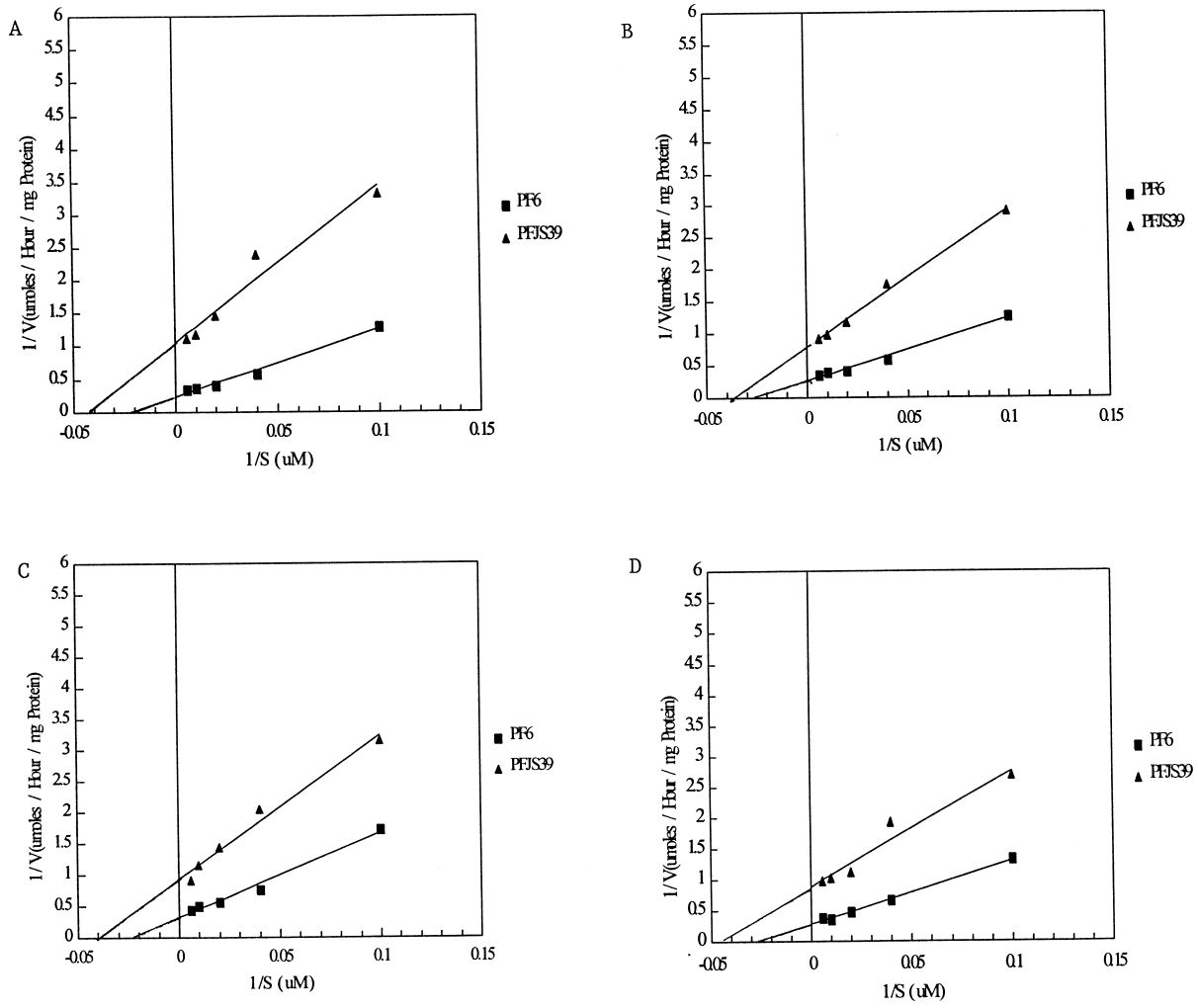

Fig. 5. Lineweaver–Burk plots of in vitro assays of 2,4-DNT conversion into MNC by whole cells and cell lysates of strains PF6 and PFJS39. (A) Whole cells grown under normal aeration; (B) lysates of cells grown under normal aeration; (C) whole cells grown under restricted aeration; (D) lysates of cells grown under restricted aeration.

Table 2
K_M and V_{max} values with respect to 2,4-DNT determined for DNT dioxygenase in whole cells and whole cell lysates of PFJS39 and PF6. Cells were grown under normal or restricted aeration as described in Section 2. All values were extracted from the data in Fig. 5

	PFJS39	PF6
K_M values (μM)		
Whole cells/normal aeration	22	45
Lysed cells/normal aeration	27	37
Whole cells/restricted aeration	25	43
Lysed cells/restricted aeration	21	36
V_{max} values (μmol / h / mg protein)		
Whole cells/normal aeration	0.94	4.31
Lysed cells/normal aeration	1.26	3.77
Whole cells/restricted aeration	1.08	3.17
Lysed cells/restricted aeration	1.12	3.48

Lineweaver–Burk plots, it was apparent that the presence of VHb increased the enzyme's V_{max} by three- to four-fold and the enzyme's K_M by 37% to 100% (Fig. 5; Table 2). There was no apparent difference in either parameter between cells grown at normal and restricted aeration.

4. Discussion

In LB with normal aeration the presence of *dntA* significantly inhibited growth. The reasons are not known but it may be related to the general growth inhibition resulting from the presence of recombinant plasmids (in this case, pJS39) [31]. In any event, similar to what has been seen in other systems [11], the presence of *vgb*/VHb alleviates about half of this growth inhibition. In minimal medium, there was a similar growth enhancement, but it occurred only under conditions of restricted aeration. Oxygen availability may also be limiting in LB at normal aeration, due to the approximately 20-fold greater growth than that which occurs in minimal medium with restricted aeration. That VHb is the reason for this growth enhancement is also supported by the experiments in which succinic acid was replaced by glucose (which is known to inhibit *vgb* expression by about 70% via catabolite repression [3]) as a carbon source. This was accompanied by a significant decrease in the advantage afforded by *vgb* at low aeration.

The conversion of 2,4-DNT to MNC could only be followed in minimal medium, because of interference of components of LB in the colorimetric assay for MNC production, but as expected from the growth data the presence of *vgb*/VHb enhanced this reaction only at restricted aeration. Actually, the 2,4-DNT to MNC conversion in both the presence and absence of *vgb* was decreased greatly under these conditions. This was due to the decrease in growth of both strains, but the decrease in 2,4-DNT catabolism was disproportionately severe in the absence of *vgb*/VHb even when the growth differences are considered. This suggests that VHb stimulates DNT dioxygenase levels and/or activity. As was mentioned above regarding growth, this is supported by the diminution in the PF6 advantage at low aeration when glucose (which inhibits *vgb* expression) was substituted for succinic acid.

The in vitro experiments using whole cells and cell extracts of strains PF6 and PFJS39 do isolate at least part of the VHb effect specifically to DNT dioxygenase, although the details of the results are not simple to interpret. Specifically, the strong (three- to four-fold) increase in the enzyme's V_{max} re: 2,4-DNT (equivalent to a three- to four-fold increase in the amount of DNT dioxygenase) is offset somewhat by a 1.4- to 2-fold increase in K_M for 2,4-DNT. The V_{max} effect could be due to either or both a VHb-stimulated increase in DNT-dioxygenase synthesis (due to increased ATP production in the host cells leading to higher synthesis of recombinant protein [7,10,12,13]) or a direct effect of VHb enhancing activity by oxygen delivery to the enzyme. The observed increase in K_M would seem to argue against oxygen delivery from VHb to the oxygenase. This result is in any case curious, but a possible explanation is that other enzymes that are indigenous to *E.*

coli and act on 2,4-DNT [20,32,33] are enhanced by the presence of VHb and compete with DNT dioxygenase for the substrate. It is also unclear why the same changes occur to the DNT dioxygenase parameters in the presence of VHb at both normal and restricted aeration, while in vivo enhancement of 2,4-DNT to MNC conversion occurs only at restricted aeration. One possibility is that the much faster growth and conversion by both strains at normal aeration obscure the direct effects on the enzyme.

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