Journal of Industrial Microbiology & Biotechnology (2001) 27, 27–33
© 2001 Nature Publishing Group 1367-5435/01 $17.00

www.nature.com/jim

Chromosomal integration of the *Vitreoscilla* hemoglobin gene in *Burkholderia* and *Pseudomonas* for the purpose of producing stable engineered strains with enhanced bioremediating ability

JW Chung[1,3], DA Webster[1], KR Pagilla[2] and BC Stark[1]

[1]*Biology Division, Department of Biological, Chemical, and Physical Sciences, Illinois Institute of Technology, IIT Center, Chicago, IL 60616;* [2]*Department of Chemical and Environmental Engineering, Illinois Institute of Technology, IIT Center, Chicago, IL 60616*

Using the pUT-miniTn*5* vector system developed by the laboratory of K.N. Timmis, the *Vitreoscilla* hemoglobin gene (*vgb*) was integrated into the chromosomes of *Pseudomonas aeruginosa* and *Burkholderia cepacia*; *Vitreoscilla* hemoglobin (VHb) was expressed at 8.8 and 0.8 nmol/g wet weight of cells in the respective engineered strains. The *vgb*-bearing *P. aeruginosa* outgrew wild-type *P. aeruginosa* and degraded benzoic acid faster than the latter strain at both normal and low aeration. In contrast, the *vgb*-bearing *B. cepacia* strain had a growth advantage over the wild-type strain at ca. 90 ppm, but not at ca. 120 ppm 2,4-dinitrotoluene (DNT); no difference in DNT degradation was seen between the two strains at either normal or low aeration. The results demonstrate the practicality of enhancing bioremediation with *vgb* stably integrated into the chromosome, but also suggest that a minimal level of VHb expression is required for its beneficial effects to be seen. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 27–33.

Keywords: *Vitreoscilla* hemoglobin; *Vitreoscilla* hemoglobin gene; chromosomal integration; bioremediation; *Burkholderia cepacia*; *Pseudomonas aeruginosa*; VHb; low oxygen expression

Introduction

Vitreoscilla hemoglobin (VHb) is an oxygen binding protein that is thought to enhance bacterial respiration at low exogenous oxygen concentrations, perhaps by binding O_2 and delivering it to the terminal oxidase [1,2]. With this possibility in mind, the VHb gene (*vgb*) has been transformed into a variety of bacteria and fungi to increase their growth and productivity [3–10]. Another aspect of this "VHb technology" is the enhancement of bioremediation by aerobic bacteria able to degrade toxic aromatic compounds oxidatively [11,12]. The bioremediation applications have utilized *vgb* transformed into heterologous hosts on a broad host range plasmid because of the ease of this technique and its possibility of supporting high VHb expression due to the gene dosage effect. For several reasons, however, such strains are not practical for actual use *in situ*. Among these are their need for antibiotic pressure to ensure phenotypic stability, possible growth disadvantages compared with plasmid-free strains, and the possible ease of horizontal transfer of the plasmid-borne antibiotic resistance genes to other species [13]. To lessen these problems and bring *vgb*/VHb-enhanced bioremediation closer to a practical technology, we report here the use of a transposon-based vector to integrate *vgb* into the chromosomes of *Pseudomonas aeruginosa* and *Burkholderia cepacia*. We also report the resultant effects on the growth of both bacteria, and degradation of benzoic acid by *P. aeruginosa* and 2,4-dinitrotoluene (DNT) by *B. cepacia*.

Correspondence: Dr BC Stark, Biology Division, Department of Biological, Chemical, and Physical Sciences, Illinois Institute of Technology, IIT Center, Chicago, IL 60616, USA

[3]Present address: Department of Pediatrics, School of Medicine, Johns Hopkins University, Baltimore, MD 21205, USA

Received 20 October 2000; accepted 4 May 2001

Materials and methods

Bacterial strains and plasmids

The bacterial strains were *P. aeruginosa* NRRL B-771, *B. cepacia* NRRL B-14180 (both obtained from Dr. L.K. Nakamura, United States Department of Agriculture, Peoria, IL), *Escherichia coli* DH5α, and *E. coli* SM10(λ*pir*) [13] (the latter obtained from Dr. K.N. Timmis, GBF-National Research Center for Biotechnology, Germany). We confirmed that B-771 and B-14180 are capable of degrading benzoic acid and DNT, respectively.

The plasmids used were pUC8:16, pUC18:*Not*I, pUT-miniTn*5*(Cm), and pUT-miniTn*5*:*vgb*(Cm) (see below). pUC8:16 contains *vgb* on a 1.4-kb fragment inserted between the *Hin*dIII and *Sal*I sites of pUC8 [14,15]. pUC18:*Not*I, a derivative of pUC18, contains *Not*I sites at both ends of the multiple cloning site of pUC18 [13]. pUT-miniTn*5*(Cm) is a transposon vector that contains the IS*50*$_R$ transposase gene and the gene for chloramphenicol (Cm) resistance [13]. Both pUC18:*Not*I and pUT-miniTn*5*(Cm) were supplied by Dr. K.N. Timmis. Small-scale purification of plasmid DNA was performed with the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI).

Pseudomonas strains were maintained on Luria broth (LB) prepared as described by Miller [16]. *Burkholderia* strains were maintained on tryptic soy agar (TSA), which was purchased from Sigma Chemical, St. Louis, MO.

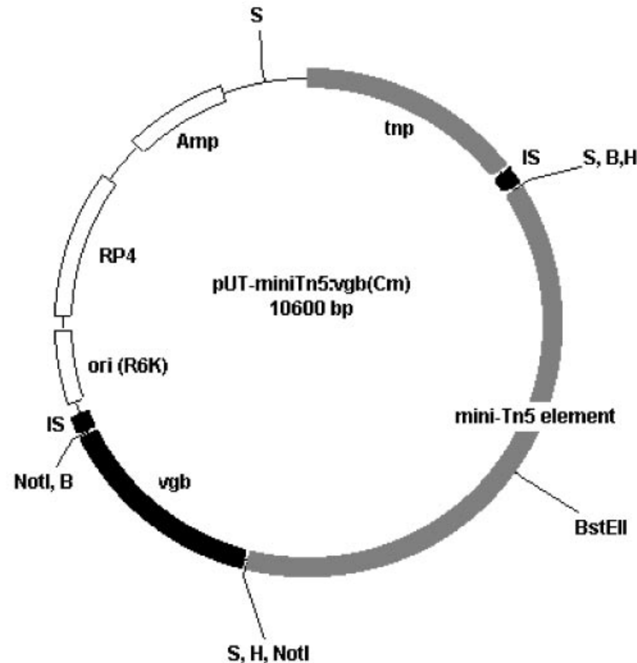


Figure 1 Restriction map of pUT-miniTn*5*:*vgb*(Cm). Positions of salient restriction sites are indicated (B, *Bam*HI; H, *Hin*dIII; S, *Sal*I). The mini-Tn*5* element and *vgb* are those sequences integrated into the chromosome (the Cm resistance gene is in the mini-Tn*5* region). Other markers are: RP4, RP4 mobilization region; ori (R6K), R6K origin of replication; IS, 19-bp Tn*5* termini; Amp, ampicillin resistance gene; tnp, transposase gene.

Construction of transposon vector, pUT-miniTn5:vgb (Cm)

The 1.4-kb *Vitreoscilla* fragment containing *vgb* was removed from pUC8:16 by restriction with *Hin*dIII and *Bam*HI, gel purified, and inserted into the same sites in the polylinker of pUC18:*Not*I. The fragment was then re-transferred by restriction with *Not*I into the *Not*I site of pUT-miniTn*5*(Cm). The ligated mixture was transformed into *E. coli* SM10(λpir) and transformants randomly selected on LB plates in the presence of ampicillin (100 μg/ml) and Cm (24 μg/ml). Transformants bearing *vgb* were identified by polymerase chain reaction (PCR) analysis (see below). The map of pUT-miniTn*5*:*vgb*(Cm) is shown in Figure 1.

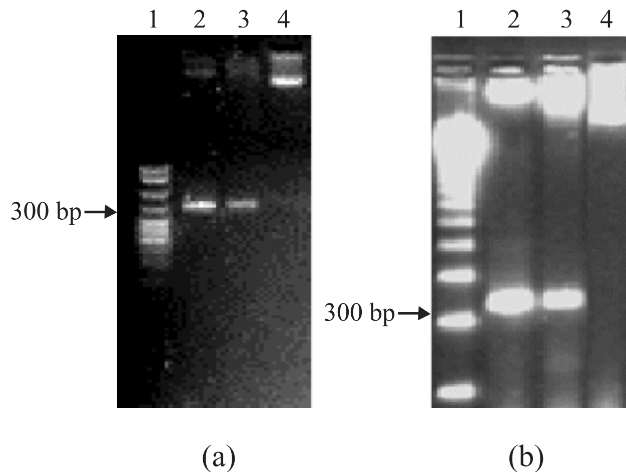


Figure 2 Confirmation by PCR of *vgb* in exconjugants. (a) *P. aeruginosa*. Lane 1, pBR322-*Msp*I digest; 2, pUC8:16; 3, PaJC chromosomal DNA; 4, PaWT chromosomal DNA. (b) *B. cepacia*. Lane 1, 123-bp ladder; 2, pUC8:16; 3, BcJC chromosomal DNA; 4, BcWT chromosomal DNA.

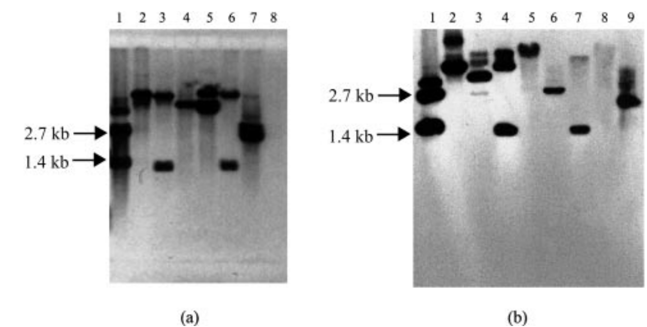


Figure 3 Southern blot analysis. (a) *P. aeruginosa*. Lane 1, pUC8:16 cleaved with *Bam*HI and *Hin*dIII; 2–4, pUT-miniTn*5*:*vgb*(Cm) cleaved with *Bst*EII, *Not*I, and *Sal*I, respectively; 5–7, PaJC chromosomal DNA cleaved with *Bst*EII, *Not*I, and *Sal*I, respectively; 8, PaWT DNA cleaved with *Sal*I. (b) *B. cepacia*. Lane 1, pUC8:16 cleaved with *Bam*HI and *Hin*dIII; 2–4, pUT-miniTn*5*:*vgb*(Cm) cleaved with *Bst*EII, *Sal*I, and *Not*I, respectively; 5–7, BcJC chromosomal DNA cleaved with *Bst*EII, *Sal*I, and *Not*I, respectively; 8, BcWT chromosomal DNA cleaved with *Sal*I; 9, *Vitreoscilla* chromosomal DNA cleaved with *Hin*dIII. In each case, the probe was pUC8:16. The ca. 9-kb signal detected from pUT-miniTn*5*:*vgb*(Cm) cleaved with *Not*I presumably represents hybridization between the probe and the Amp gene in the pUT-miniTn*5*(Cm) vector.

Transposon-mediated transfer of vgb

Transfer of pUT-miniTn*5*:*vgb*(Cm) from *E. coli* SM10(λpir) [pUT-miniTn*5*:*vgb*(Cm)] to *B. cepacia* and *P. aeruginosa* was performed by conjugation using a procedure modified from that of Herrero *et al.* [13]. *B. cepacia* and *P. aeruginosa* exconjugants were selected on TSA containing kanamycin (Km) (100 μg/ml) and Cm (400 μg/ml), and LB containing Km (100 μg/ml) and Cm (500 μg/ml), respectively.

Polymerase chain reaction

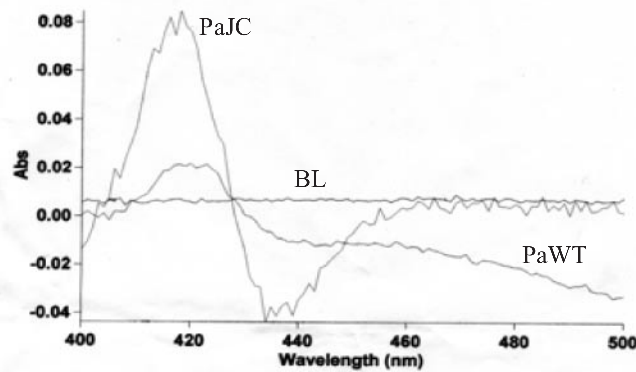
Purification of genomic DNA of the exconjugants was performed with the Wizard Genomic DNA Purification System (Promega). On the basis of the known sequence of *vgb*, forward (left) and reverse (right) primers were designed to amplify a 300-bp internal portion of *vgb*. In a 0.5-ml thin-wall tube, the following components were combined: 2 μl of 10×PCR-reaction buffer (100 mM Tris–HCl, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 μl of dNTP mix (10 mM each: dATP, dCTP, dGTP, dTTP), 1 μl of template DNA (10 μg/μl), 0.2 μl (0.13 μg) of primer 5′CATCAAAGCCACTGTTCCTGT3′ (left), 0.2 μl (0.13 μg) of primer 5′CTTCTTTAATCGCACCCAACA3′ (right), 0.2 μl of Taq DNA polymerase (Promega, 5 units/μl), and 16.2 μl of H_2O. Twenty microliters of mineral oil was overlaid to reduce evaporation during the amplification. Amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer, Wellesley, MA) for 35 cycles, each cycle being 30 s at 95°C (denaturation), 30 s at 55°C (hybridization), and 30 s at 72°C (elongation).

Southern hybridization

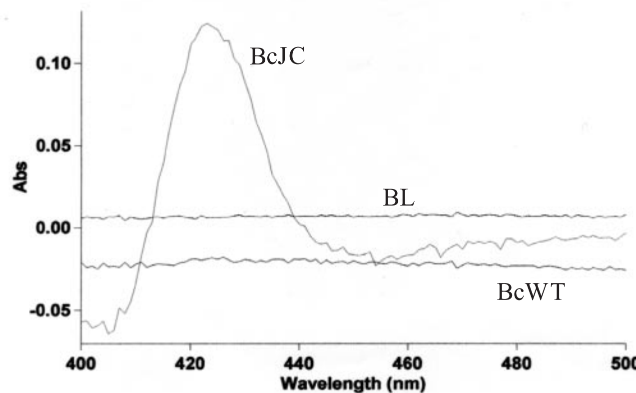
Genomic DNA samples (10 μg) were digested with restriction enzymes, and the resulting fragments were separated according to size by electrophoresis through 1% agarose gels in 1×TBE buffer [17]. The DNA was then denatured *in situ* and transferred by capillary action from the gel to Hybond-N membrane (Amersham, Uppsala, Sweden). Labeling of the probe (pUC8:16) and hybridization were performed with the Enhanced Chemiluminescence system (Amersham) according to the manufacturer's protocol.

CO-difference spectral analysis

Strains that were positive on the Southern blot were analyzed by CO-difference spectra using either whole cells or cytoplasm



(a)



(b)

Figure 4 CO-difference spectra. (a) Whole cell spectra of PaWT and PaJC. The concentration was 50 mg wet weight/ml in each case. The baseline (BL) was for PaJC (the PaWT baseline was similar). (b) Spectra of cytoplasm of BcWT and BcJC. Samples were diluted with 20 mM potassium phosphate, pH 7.2, to a protein concentration of 30 mg/ml. The baseline was for BcJC (the BcWT baseline was similar).

[18,19] and a Cary 300 scanning spectrophotometer (Varian, Walnut Creek, CA) to confirm that VHb was produced in these strains. For whole cell analysis, *Pseudomonas* and *Burkholderia* strains were incubated in LB or tryptic soy broth (TSB), respectively, in shake flasks overnight at 30°C, and samples were taken from stationary phase cultures.

For cytosolic analysis of *B. cepacia*, cells were grown to stationary phase at 30°C in 1-l flasks containing 500 ml of TSB. Cytosol was prepared by a modification of the method of Abrams and Webster [20]. The cytosolic fractions were diluted with 20 mM potassium phosphate, pH 7.2, to protein concentrations of 30 mg/ml (determined by absorbance at 280 nm) and used directly for CO-difference spectral analysis as described above. VHb levels were calculated using the extinction coefficient $E_{419-436\ \mathrm{nm}}$=274 $\mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$ [14].

Growth comparisons

Shake flask cultures of strains (either *vgb*-free or *vgb*-bearing) to be used as inocula were grown in 25 ml of LB or TSB as required for 12 h at 30°C and 200 rpm. Approximately 0.1 ml of each culture (volume adjusted to give a constant A_{600} within each experiment, varying from 0.017 to 0.023) was harvested by centrifugation, and inoculated into 50 or 200 ml of medium after being washed twice with the same medium at 30°C. These media included, for *P. aeruginosa*, LB and M9 [16] supplemented with 1000 ppm benzoic acid (adjusted to pH 7.4 before addition) and, for *B. cepacia*, TSB initially containing 120 or 180 ppm DNT. In the case of the TSB–DNT medium, the actual DNT concentrations measured at the time of inoculation were 88–94 and 119–125 ppm, respectively. The reason for the differences is not known, but may be related to autoclaving of the medium. TSB supplemented with DNT was used in these experiments because a preliminary experiment showed that *B. cepacia* grew very slowly in M9 medium with DNT as sole carbon source.

Normal aeration was 200 rpm in Erlenmeyer flasks in which the medium volume was 20% of the flask volume; low aeration was 50 rpm in Erlenmeyer flasks in which the medium volume was 80% of the flask volume. The numbers of viable cells were determined by plating dilutions (in 0.85% NaCl) on LB in triplicate. Colonies were counted after growth for 16 h at 30°C. At each time point, the A_{600} of each culture was also determined; measurements were kept below 0.5 by dilution with fresh medium as necessary. *P. aeruginosa* experiments in LB and M9-benzoate lasted 144 and 72 h, respectively. Experiments with *B. cepacia* lasted 72 h.

Measurement of benzoic acid and DNT levels

At each time point during growth in media containing benzoate or DNT, cells were removed by centrifugation and supernatant fluids were analyzed by HPLC (Varian Star Chromatography 9012Q solvent delivery system with 9050 UV–Vis detector) using a C18 reverse phase column with an elution gradient of 25% acetonitrile/75% aqueous trifluoroacetic acid changed to 50% acetonitrile/50% aqueous trifluoroacetic acid over 10 min. The flow rate was 1.5 ml/min and all compounds were detected by absorbance at 230 nm. Absolute values were determined by comparison of peak areas with peak area versus ppm standard curves for each compound; each standard curve was linear over the entire concentration range tested in the growth experiments.

Results

Confirmation by PCR of successful conjugation

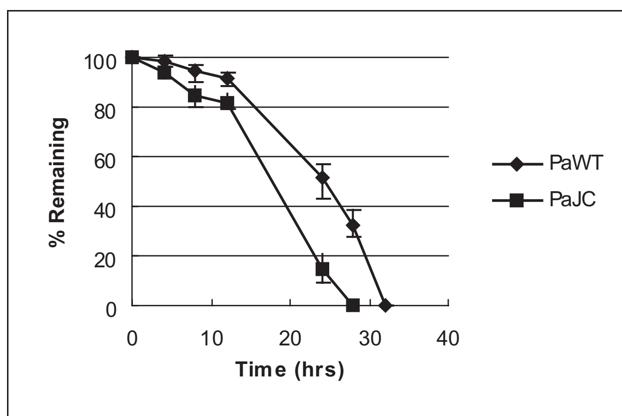
To identify *P. aeruginosa* containing *vgb*, total DNA was extracted from each of 32 colonies that grew on the selective medium following conjugation. Using these DNA preparations as templates, PCR was performed. Nineteen colonies gave products of about 300 bp and thus were candidates to have *vgb* integrated into their chromosomes. One of these was selected for further experiments and named *P. aeruginosa* JC (abbreviated PaJC) (Figure 2a). Among five colonies of *B. cepacia* selected after conjugation, only one showed the expected 300-bp *vgb* amplification product (Figure 2b). This colony was chosen for further experiments and named *B. cepacia* JC (abbreviated BcJC). The corresponding wild-type strains were named PaWT and BcWT.

Southern blot analysis confirming successful chromosomal integration of vgb

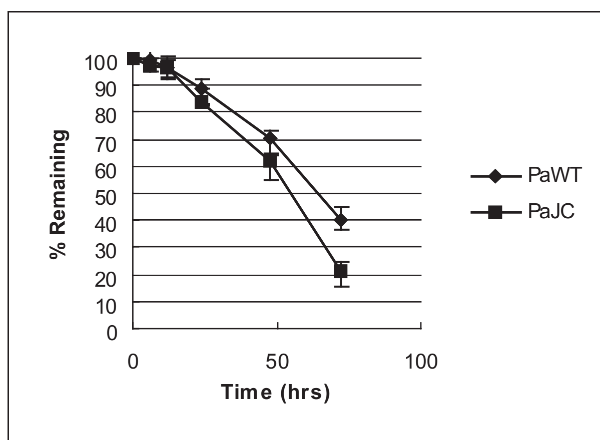
The PCR results described above proved the presence of *vgb* in the exconjugants, but not its chromosomal integration. This was confirmed by Southern hybridization. Chromosomal DNA was isolated from PaJC and BcJC as well as each wild-type and electrophoresed on 1% agarose gels after complete digestion with *Sal*I, *Bst*EII, and *Not*I. Control plasmid pUT-miniTn*5*:*vgb*(Cm) was also electrophoresed on the same gels after digestion with the same enzymes to compare its patterns with those of the chromosomal samples. In the Southern blots using pUC8:16 as probe, positive signals occurred at 10.6 kb for the *Bst*EII cut, 5.1 kb for the *Sal*I cut, and 1.4 kb for the *Not*I cut for the control plasmid (Figure 3). These were expected from the restriction map (Figure 1). The signals from the chromosomal DNA of PaJC were at approximately 8.0 kb for the *Bst*EII cut, 2.5 kb for the *Sal*I cut, and 1.4 kb for the *Not*I cut (Figure 3a). The signals from the

Table 1 Summary of growth experiments. Both maximum A_{600} and viable cell counts (colony forming units/ml) are listed. All values are averages of three individual experiments; standard deviations (σ_{n-1}) are in parentheses. All maxima occurred in stationary phase except for the maximum A_{600}s for PaWT and PaJC at low aeration, which occurred in death phase. MB is M9-benzoate medium

Strain	Medium	Aeration	Max. A_{600}	Max. viable
PaWT	LB	Normal	3.42 (0.26)	11 (1) E9
		Low	2.50 (0.07)	1.8 (0.3) E9
	MB	Normal	0.44 (0.02)	3.4 (0.8) E9
		Low	0.37 (0.02)	0.16 (0.06) E9
PaJC	LB	Normal	3.45 (0.31)	12 (3) E9
		Low	2.68 (0.04)	2.7 (0.4) E9
	MB	Normal	0.45 (0.03)	4.0 (0.7) E9
		Low	0.42 (0.02)	0.37 (0.08) E9
BcWT	TSB/ 88–94 ppm DNT	Normal	3.52 (0.26)	6.8 (0.4) E9
		Low	0.26 (0.02)	0.042 (0.003) E9
BcJC	TSB/ 88–94 ppm DNT	Normal	3.85 (0.32)	7.2 (0.6) E9
		Low	0.30 (0.01)	0.051 (0.004) E9
BcWT	TSB/ 119–125 ppm DNT	Normal	2.02 (0.17)	4.6 (0.6) E9
		Low	0.14 (0.00)	0.041 (0.005) E9
BcJC	TSB/ 119–125 ppm DNT	Normal	2.06 (0.08)	4.5 (0.4) E9
		Low	0.14 (0.00)	0.046 (0.005) E9



(a)



(b)

Figure 5 Benzoic acid degradation by PaWT and PaJC in M9 medium containing 1000 ppm benzoic acid. (a) Normal aeration. (b) Low aeration. All points are the averages of three individual experiments; error bars indicate standard deviations (σ_{n-1}). 100% represents 1000 ppm benzoic acid.

chromosomal DNA of BcJC were at larger than 24 kb with *Bst*EII, 3.0 kb with *Sal*I, and 1.4 kb with *Not*I (Figure 3b). For *Sal*I and *Bst*EII, signals were located at different locations for the plasmid and the chromosomal DNA from both species. This is consistent with a nonplasmid (i.e., chromosomal) location for *vgb* in both cases. For *Not*I digestions, the signals were at the same locations for plasmid and both chromosomal DNAs because there are *Not*I restriction sites at both ends of the 1.4-kb *vgb* fragment in both plasmid and chromosomally integrated forms. The other controls were as expected for this interpretation with chromosomal DNA from neither PaWT nor BcWT showing any signal, while hybridization occurred to the expected 2.3-kb *Hin*dIII fragment of *Vitreoscilla* DNA.

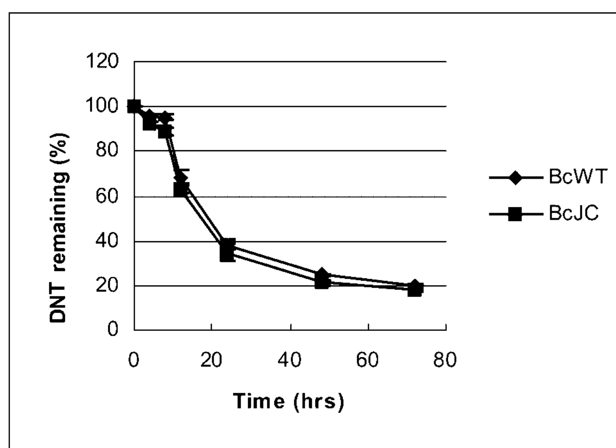
CO-spectral analysis

Whole cells of PaJC showed a typical VHb spectrum in which the peak was at 419 nm and the trough at 436 nm (Figure 4a). The whole cell spectrum of BcJC also showed an apparent VHb peak at 419 nm, but without a distinguishable trough (data not shown). However, with the cytosolic fraction of BcJC, a more obvious VHb peak was observed (Figure 4b). The VHb level for PaJC was 8.8 and for BcJC, 0.8 nmol/g wet weight of cells. Although PaWT yielded a CO-difference spectrum with a broad peak centered at about 420 nm, neither PaWT nor BcWT gave a typical VHb spectrum. With the results of these experiments, the expression of VHb in the recombinant strains was confirmed.

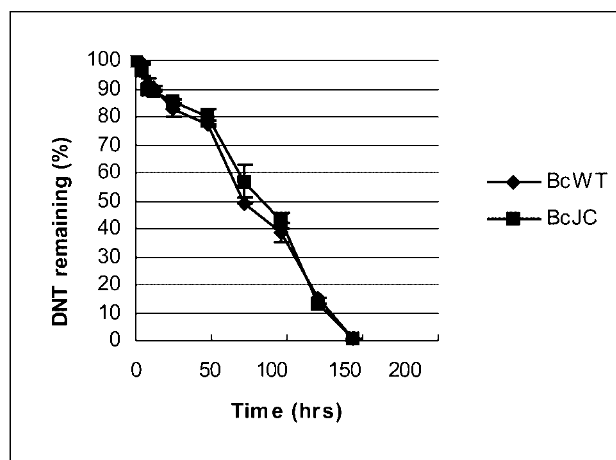
Growth comparisons at normal and limited aeration

Growth data for PaJC and BcJC as well as the corresponding wild-type strains are summarized in Table 1.

At normal aeration in LB, PaJC exhibited no significant growth advantage over PaWT as measured by A_{600}, but PaJC had slightly higher viable cell numbers than PaWT by stationary phase. In M9-



(a)



(b)

Figure 6 DNT degradation by BcWT and BcJC in TSB medium containing approximately 120 ppm DNT. (a) Normal aeration. (b) Low aeration. All points are averages of three individual experiments; error bars indicate standard deviations (σ_{n-1}). 100% represents 119–125 ppm DNT, the actual concentrations measured at time zero.

benzoate medium at normal aeration, growth of the two strains was similar in terms of A_{600}, but PaJC had greater viability than PaWT after stationary phase was reached.

At low aeration in LB, PaJC had a significant advantage over PaWT in terms of both A_{600} and viable cell numbers, but only after late log phase. In M9-benzoate medium at low aeration, PaJC exhibited a significant advantage over PaWT from mid-log phase on with regard to both A_{600} and viable cell numbers.

In TSB containing 88–94 ppm of DNT at normal aeration, the A_{600} of BcJC was higher than that of BcWT at all phases; viable cell counts for both strains were similar with BcJC higher at the last time point sampled (in stationary phase). At normal aeration with 119–125 ppm of DNT, growth of both strains was almost the same in terms of both A_{600} and viable cell counts throughout the growth curve.

With low aeration, BcJC exhibited better growth than BcWT at all phases regarding both A_{600} and viable cell numbers at 88–94 ppm DNT; there was, however, only a small advantage of BcJC at the higher DNT concentration (again at all phases). The concentration of DNT did not have much affect on the viability of the strains under low aeration conditions; at both DNT concentrations, both strains were still in stationary phase when the experiments ended at 72 h.

Benzoate degradation by PaWT and PaJC

When PaWT and PaJC were grown with normal aeration in shake flasks in M9 medium supplemented with 1000 ppm of benzoic acid, the presence of *vgb*/VHb afforded a consistent advantage regarding benzoate degradation. For example, at normal aeration, PaJC degraded 85% and PaWT 50% of the benzoate by the end of log phase (24 h) (Figure 5a). The same trend occurred at low aeration although the relative advantage of PaJC was slightly less than at normal aeration (Figure 5b).

DNT degradation by BcWT and BcJC

For an initial DNT concentration of 88–94 ppm and normal aeration, the patterns of DNT degradation were essentially identical for both BcWT and BcJC at all times (data not shown), and similar results were obtained at 119–125 ppm DNT (Figure 6a). At both DNT concentrations and low aeration, the patterns of DNT degradation were essentially identical for both strains (shown for 119–125 ppm DNT in Figure 6b; at 119–125 ppm DNT, DNT degradation was followed for 144 h instead of 72 h, since only about 50% of the DNT was degraded in 72 h).

Discussion

The fact that PCR using *vgb*-specific primers amplified fragments of the expected length from both the *P. aeruginosa* and *B. cepacia* exconjugants, but not the respective wild-type strains, confirms that *vgb* was successfully transferred into each bacterium. The π protein is required for replication of the pUT-miniTn*5* series [13], and we did not expect the two host species to have this function. Thus, the supposition is that *vgb* has been integrated into the host chromosome in each case (integration of the Tn*5* module from pUT-miniTn*5* has been shown to occur in the related species *P. putida* [21]). That this is in fact the case in our experiments is supported by changes in the restriction fragment sizes detected by a *vgb*-specific probe for both *P. aeruginosa* and *B. cepacia* exconjugants compared with those detected from the donor plasmid.

The level of VHb in PaJC (8.8 nmol/g wet weight of cells) is very similar to the levels in engineered *P. aeruginosa*, *P. putida*, *Xanthomonas maltophilia* [22], and *B. cepacia* [12] in which *vgb* is carried on the pKT230-derived plasmid pSC160. It is also reasonably close to the 24–26 nmol/g wet weight reported for *P. putida* and *P. aeruginosa* bearing *vgb* on vector pVDX18 and grown under similar conditions [23]. pKT230 is reported to be a high copy number vector in *P. aeruginosa* [24], and we have measured pSC160 at about 15 copies per cell in *B. cepacia* [12]. It is unclear why VHb is expressed at essentially the same level in *P. aeruginosa* bearing pSC160 and PaJC, with a presumed *vgb* copy number of one.

A VHb level of 8.8 nmol/g wet weight is about 5% and 30% of the induced levels in *E. coli* and *Vitreoscilla*, respectively [14]. The levels in *E. coli* are correlated with increases in growth and protein production (as discussed above), and that in *Vitreoscilla* presumably reflects the normal VHb level for enhancing growth at low oxygen levels. Nevertheless, VHb between 7.4 and 12.6 nmol/g wet weight enhanced growth in *P. aeruginosa*, *X. maltophilia*, and *B. cepacia* [12,22], degradation of benzoic acid by *X. maltophilia* under some conditions [11], and degradation of DNT by *B. cepacia* [12]. Thus, it is not surprising that for PaJC, growth and benzoate degradation are greater than for PaWT. That the growth advantage of PaJC is greatest under low aeration is reminiscent of our results with *E. coli* in which *vgb*/VHb enhanced recombinant protein production only under conditions of oxygen limitation [7]. It is unclear, however, why this trend did not also occur for benzoate degradation.

In BcJC, the level of VHb expression is very low and, in contrast to the case of PaJC, approximately linearly related to the gene dosage when compared with *B. cepacia* bearing pSC160 [12]. It is possible that sequences needed for *vgb* expression (the *Vitreoscilla* promoter, Fnr and CAP binding sites, Shine-Dalgarno sequence, etc. present in the cloned *vgb* sequence [23]) are not recognized efficiently in *B. cepacia*. This low level of expression is apparently not enough to afford significant growth and DNT degrading advantages for the *vgb*-bearing strain.

As discussed above, VHb levels of about 10 nmol/g wet weight in heterologous hosts appear to be enough to elicit increased growth and biodegradation. What the lowest level of VHb expression is that can do this and whether higher concentrations can enhance the positive effects are not yet known. In any event, the ability to integrate functional *vgb* into the chromosomes of heterologous hosts is an important step in taking VHb technology applied to bioremediation into the field. It is apparent, however, that further engineering of the *vgb* control sequences, presumably on a case-by-case basis, will be needed to optimize these systems.

Acknowledgements

This work was supported in part by National Science Foundation grant number BES-9309759, Air Force Office of Scientific Research grant number F49620-95-1-0325, and an Education and Research Initiative Fund grant from the Illinois Institute of Technology. We thank Dr. K.N. Timmis for supplying plasmids pUC18:*Not*I and pUT-miniTn*5*(Cm) as well as *E. coli* strain SM10(λpir).

References

1 Wakabayashi S, H Matsubara and DA Webster. 1986. Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. *Nature* 322: 481–483.

2 Tsai PS, M Nageli and JE Bailey. 1996. Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. *Biotechnol Bioeng* 49: 151–160.

3 Khosla C and JE Bailey. 1988. Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli*. *Nature* 331: 633–635.

4 Khosravi M, DA Webster and BC Stark. 1990. Presence of the bacterial hemoglobin gene improves α-amylase production of a recombinant *Escherichia coli* strain. *Plasmid* 24: 1–5.

5 Magnolo SK, DL Leenutaphong, JA DeModena, JE Curtis, JE Bailey, JL Galazzo and DE Hughes. 1991. Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin. *Bio/Technology* 9: 473–476.

6 DeModena JA, S Gutierrez, J Velasco, FJ Fernandez, RA Fachini, JL Galazzo, DE Hughes and JF Martin. 1993. The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. *Bio/Technology* 11: 926–929.

7 Buddenhagen RE, DA Webster and BC Stark. 1996. Enhancement by bacterial hemoglobin of amylase production in recombinant *E. coli* occurs under conditions of low O_2. *Biotechnol Lett* 102: 695–700.

8 Kallio PT and JE Bailey. 1996. Intracellular expression of *Vitreoscilla* hemoglobin (VHb) enhances total protein secretion and improves the production of α-amylase and neutral protease in *Bacillus subtilis*. *Biotechnol Prog* 12: 31–39.

9 Tari C, SJ Parulekar, BC Stark and DA Webster. 1998. Synthesis and excretion of α-amylase in *vgb*+ and *vgb*− recombinant *E. coli*: a comparative study. *Biotechnol Bioeng* 59: 673–678.

10 Enayati N, SJ Parulekar, BC Stark and DA Webster. 1999. Production of α-amylase in fed-batch cultures of *vgb*+ and *vgb*− recombinant *E. coli*: some observations. *Biotechnol Prog* 15: 640–645.

11 Liu SC, DA Webster, ML Wei and BC Stark. 1996. Genetic engineering to contain the *Vitreoscilla* hemoglobin gene enhances degradation of benzoic acid by *Xanthomonas maltophilia*. *Biotechnol Bioeng* 49: 101–105.

12 Patel SM, BC Stark, KW Hwang, KL Dikshit and DA Webster. 2000. Cloning and expression of the *Vitreoscilla* hemoglobin gene in *Burkholderia* sp. strain DNT for enhancement of 2,4-dinitrotoluene degradation. *Biotechnol Prog* 16: 26–30.

13 Herrero M, V de Lorenzo and KN Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* 172: 6557–6567.

14 Dikshit KL and DA Webster. 1988. Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. *Gene* 70: 377–386.

15 Liu SC, XY Liu, DA Webster and BC Stark. 1994. Sequence of the region downstream of the *Vitreoscilla* hemoglobin gene: *vgb* is not part of a multigene operon. *Appl Microbiol Biotechnol* 42: 304–308.

16 Miller JP. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

18 Liu CY and DA Webster. 1974. Spectral characteristics and interconversions of the reduced, oxidized and oxygenated forms of purified cytochrome *o*. *J Biol Chem* 244: 4261–4266.

19 Georgiou CD and DA Webster. 1987. Purification and partial characterization of the membrane-bound cytochrome *o*(561,564) from *Vitreoscilla*. *Biochemistry* 26: 6521–6526.

20 Abrams JJ and DA Webster. 1990. Purification, partial characterization and possible role of catalase in the bacterium *Vitreoscilla*. *Arch Biochem Biophys* 279: 54–59.

21 De Lorenzo V, M Herrero, U Jakubzik and KN Timmis. 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J Bacteriol* 172: 6568–6572.

22 Liu SC, DA Webster and BC Stark. 1995. Cloning and expression of the *Vitreoscilla* hemoglobin gene in *Pseudomonas*: effects on cell growth. *Appl Microbiol Biotechnol* 44: 419–424.

23 Joshi M and KL Dikshit. 1994. Oxygen dependent regulation of *Vitreoscilla* globin gene: evidence for positive regulation by FNR. *Biochem Biophys Res Commun* 202: 535–542.

24 Bagdasarian M, R Lurz, B Ruckert, FCH Franklin, MM Bagdasarian, J Frey and KN Timmis. 1981. Specific-purpose plasmid cloning vectors: II. Broad host range, high copy number, RSF1010-derived vectors and a host–vector system for gene cloning in *Pseudomonas*. *Gene* 16: 237–247.