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Enhanced Biodegradation of Environmental Allergen by a *vgb*-containing *Burkholderia cepacia*

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Abstract

Using genetic engineering, the *Vitreoscilla* (bacterial) hemoglobin gene (*vgb*) was integrated stably into the chromosomes of and *Burkholderia cepacia*. Similar to previous results, the wild type VHb improved growth for *Burkholderia cepacia* and degradation of benzoic acid under both normal and low aeration conditions. The *stable* expression of VHb enhanced these parameters. The results demonstrate the possibility that the positive effects provided by VHb may be augmented by protein engineering.

Keywords: biodegradation, environmental allergen, benzoic acid, bacterial hemoglobin

Environmental contamination by industrial, military and municipal pollutants is a serious problem worldwide. Human exploitation of fossil fuel reserves and the production of many novel synthetic compounds in the twentieth century have introduced into the environment many compounds that microorganisms normally do not encounter and thus are not prepared to biodegrade. Many of these compounds are toxic to living systems and their presence in aquatic and terrestrial habitats often has serious ecological consequences, including major kills of indigenous biota. The disposal or accidental spillage of these compounds creates serious modern environmental pollution problems, particularly when microbial biodegradation activities fail to remove the pollutants quickly enough to prevent environmental damage. Indigenous bacteria found in contaminated environments frequently possess the biochemical potential to degrade many of these toxic compounds. Members of the genus *Pseudomonas* are the most predominant group of soil microorganisms that degrade xenobiotic compounds. In many cases, one strain can use several different related compounds as a sole carbon source. However, their biological activities are often quite slow. Nevertheless, the use of microorganisms to remediate sites contaminated with hazardous wastes is becoming an increasingly popular environmental clean up technique. These microorganisms can be selected from the field with enrichment culture media containing the toxic product as the sole source of carbon for growth[1].

It has been demonstrated, through genetic engineering, that intracellular expression of a bacterial hemoglobin from *Vitreoscilla* (VHb) in different hosts elicits, *in vivo*, effects of reduced oxygen starvation, improved cell growth, and increased formation of valuable products[2-5]. The VHb gene (*vgb*) has also been inserted into several members of the *Pseudomonadaceae* on a recombinant plasmid for the purpose of enhancing their ability to degrade toxic aromatic compounds[6]. Recently, we have stably integrated *vgb* into the chromosome of *Pseudomonas aeruginosa* and found that this *vgb*-containing strain still maintains enhanced growth and benzoic acid degradation[7].

Benzoic acid is the most common environmental pollutant that causes several allergic symptoms including urticaria[8], asthma[9], and persistent rhinitis[10]. Furthermore, it is itself an intermediate in the breakdown of several aromatic pollutants. Previously, we have constructed *vgb*-containing *Burkholderia cepacia* strain on the purpose of efficient biodegradation of 2, 4-dinitrotouleue (DNT) using transposon-mediated gene transfer[7]. However, the strain was not able to significantly enhance the biodegrading capacity against DNT, although *vgb* was successfully integrated into the chromosome and stably expressed.

It might due to the specificity of the toxicant that can be degraded by the microorganism. Thus in this report, we have examined the biodegrading capacity of *Burkholderia cepacia* against benzoic acid, which is a potent and common allergen or toxicant in the environment.

PCR Analysis of Existence of *vgb* in Exconjugant

To identify and confirm existence of *vgb* in *B. cepacia* JC (BcJC), total DNA was extracted from the colony that grew on the selective medium following conjugation. Using these DNA as template, PCR was performed. As expected, the colony showed 300-bp *vgb* amplification product, while wild-type strain of *B. cepacia* (BcWT) had no amplified product (Fig. 1A).

Southern Blot Analysis Confirming Successful Integration of *vgb* into the Chromosome

Although the presence of *vgb* was confirmed by PCR analysis, it did not prove its chromosomal integration. Thus we next performed the Southern hybridization to prove successful chromosomal integration of *vgb*. Chromosomal DNA was isolated from BcJC as well as BcWT and electrophosresed on 1% agarose gels after complete digestion with *SalI*, *BstEII*, and *NotI*. Control plasmid pUT-miniTn5 : *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. When pUC8 : 16 was used as the probe, positive signals occurred at 10.6 kb for the *BstEII* cut, 5.1 kb for the *SalI* cut, and 1.4 kb for the *NotI* cut for the control plasmid (Fig. 1B). The signals from the chromosomal DNA of BcJC were at larger than 24 kb with *BstEII*, 3.0 kb with *SalI*, and 1.4 kb with *NotI* (Fig. 1B). For *SalI* and *BstEII*, signals were located at different locations for the plasmid and the chromosomal DNA from the exconjugant. For *NotI* digestion, the signal was at the same locations for plasmid and chromosomal DNA because there are *NotI* restriction sites at both ends of the 1.4-kb *vgb* fragment in both plasmid and chromosomally integrated forms. The other control was as expected for this interpretation with chromosomal DNA from BcWT showing no signal, while hybridization occurred to the expected 2.3-kb *HindIII* fragment of *vgb*.

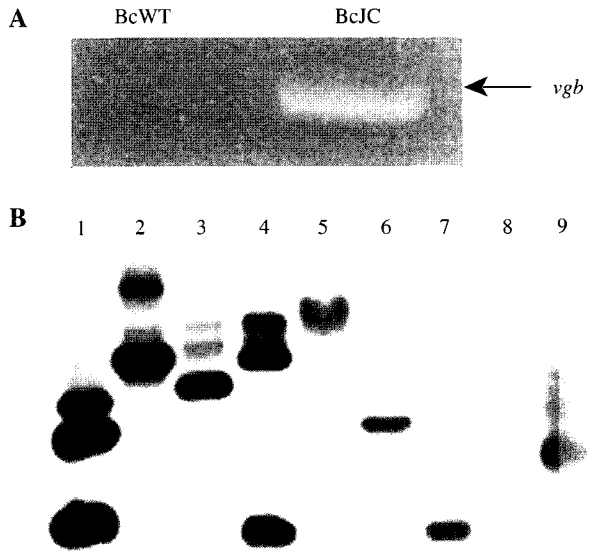

Fig. 1. Confirmation of successful integration of *vgb* in the chromosome of *Burkholderia cepacia*. (a) PCR analysis of *vgb* using chromosomal DNAs of BcWT (lane 1) and BcJC (lane 2). (b) S. blot analysis. Lane 1, pUC8 : 16 cleaved with *BamHI* and *HindIII*; 2-4, pUT-miniTn5 : *vgb* (Cm) cleaved with *BstEII*, *SalI* and *NotI*, respectively; 5-7, BcJC chromosomal DNA cleaved with *BstEII*, *SalI*, and *NotI*, respectively; 8, BcWT chromosomal DNA cleaved with *SalI*; 9, *Vitreoscilla* chromosomal DNA cleaved with *HindIII*. In each case, the probe was pUC8 : 16.

Spectrophotometric Analysis of VHb Expression

In previous work, it has been shown that expression of VHb can be identified spectrally using CO-difference spectra of intact cells or cell extracts at room temperature[12]. In such spectra VHb has a characteristic absorption maximum at 419 nm and minimum at 436-7 nm. Based on the above information, the recorded spectra of the strains were analyzed. Whole cells and the cytosolic fraction of BcJC showed a typical VHb spectrum in which the peak was at 419 nm and the trough at 436 nm, while BcWT did not

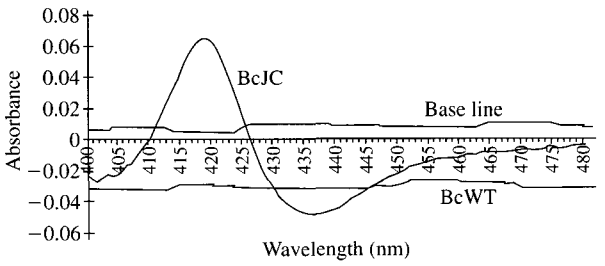

Fig. 2. CO-difference spectral analysis. Cytoplasmic spectra of BcWT and BcJC. Samples were diluted with 20 mM potassium phosphate, pH 7.2, to a protein concentration of 30 mg/mL.

Table 1. Growth of untransformed *Burkholderia cepacia* compared with those of the corresponding transformants bearing the *Vitreoscilla* (bacterial) hemoglobin gene (*vgb*)

Strain	Normal aeration		Low aeration	
	Maximum OD_{600}	Maximum viable cells	Maximum OD_{600}	Maximum viable cells
BcWT	3.01	2.10 E10	0.82	1.82 E9
BcJC	3.37	2.98 E10	1.91	2.67 E10

All values are averages of 2-3 independent measurements. Standard deviations ranged from 2 to 6%, 7-18%, and 2-9% of averages for OD_{600}, viable cells, and degradation measurements, respectively

Table 2. Growth comparisons of *Burkholderia* bearing *vgb* in minimal medium containing benzoic acid as a soul carbon source

Growth (OD_{600})		Growth (viable cells)	
Normal aeration			
Strain		Strain	
BcWT	3.55 (100)	BcWT	2.73 E10 (100)
BcJC	3.70 (104)	BcJC	3.89 E10 (142)
Low aeration			
Strain		Strain	
BcWT	1.36 (100)	BcWT	2.34 E9 (100)
BcJC	1.54 (113)	BcJC	6.62 E9 (283)

All values are maximum values achieved (averages of 2-3 independent measurements), with relative values in parentheses. Standard deviations ranged from 0.5 to 4%, and 2-26% of averages for OD_{600} and viable cells, respectively

give a typical VHb spectrum (Fig. 2). With the results of this experiment, the expression of VHb in the recombinant strains was confirmed.

Growth Comparison of *B. cepacia* and *B. cepacia* JC at Normal and Restricted Aeration

When *Burkholderia* strains were compared regarding growth in rich medium (TSB), the strain bearing wild type *vgb* outgrew the untransformed strain regarding both maximum OD_{600} and maximum viable cells under both normal aeration and hypoxic conditions (Table 1). When the *Burkholderia* transformants were compared regarding growth at both normal and low aeration in minimal medium with benzoic acid, there was a slight advantage regarding OD_{600} of the strains bearing the mutant *vgb*, but only at low aeration; viability was increased for the mutants at both aerations, but more so at low aeration (Table 2).

Benzoate Degradation by BcWT and BcJC

When BcWT and BcJC were grown with normal aeration in shake flasks in minimal medium containing 1,000 ppm of benzoic acid, the presence of *vgb*/VHb afforded a consistent advantage regarding benzoate degradation. For example, at normal aeration, BcJC degraded 85% and BcWT 50% of the benzoate by the end of log phase (24 h) (Fig. 3a). The same trend occurred at restricted aeration although the relative advantage of BcJC was slightly less than at normal aeration (Fig. 3b).

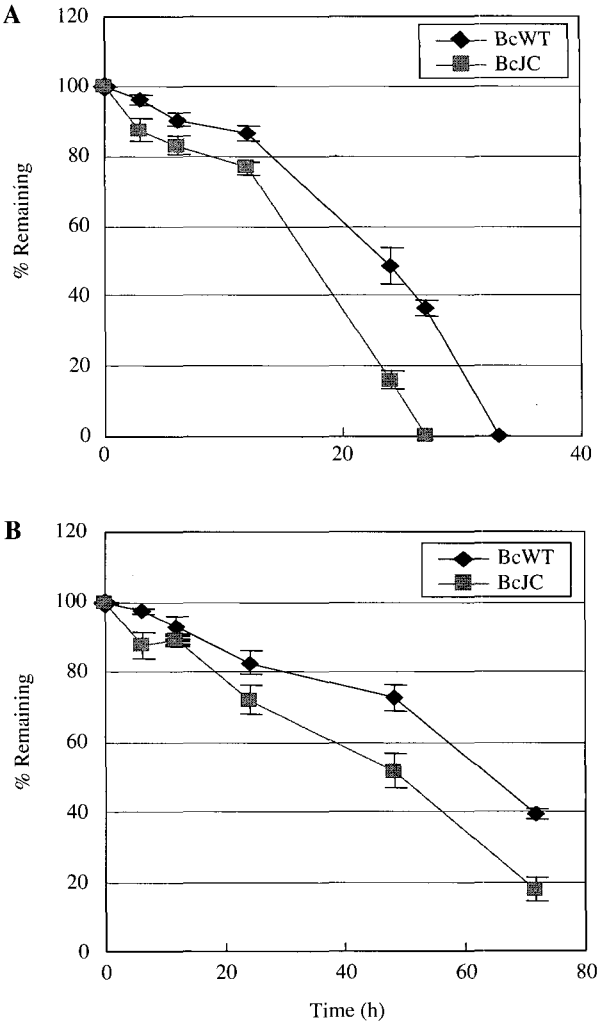

Fig. 3. Benzoic acid degradation by BcWT and BcJC in minimal medium with 1,000 ppm benzoic acid. (a) Normal aeration. (b) Restricted aeration. All points are the averages of three individual experiments. 100% represents 1,000 ppm benzoic acid.

Discussion

The PCR result using *vgb*-specific primers amplifi-

ed fragments of the expected length from *B. cepacia* exconjugants, but not the wild-type strain, confirms that *vgb* was successfully transferred into the bacterium, and the S. blot analysis proved that the gene was present in the chromosome of the bacterium, not in the form of plasmid, in which the changes in the restriction fragment sizes were detected by a *vgb*-specific probe for *B. cepacia* exconjugants compared with those detected from the donor plasmid. The level of VHb in BcJC, calculated from the CO-spectral analysis, was 9 nmol/g wet weight of cells. It has been reported that the VHb levels in recombinant E. coli are correlated with increases in growth and protein production[13]. Thus, it is not surprising that for BcJC, growth and benzoate degradation are greater than for BcWT. It is possible that the ability of the recombinant strain to maintain the ability to degrade benzoic acid 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.

Methods

Bacterial Strains, Plasmids and Media

The bacterial strains were *B. cepacia* NRRL B-14180, *E. coli* DH5a, and E. coli SM10 (λ*pir*). The plasmids used were pUC8:16, pUC18 : NotI, and pUT-miniTn5 (Cm)[7]. *Burkholderia* strains were maintained on tryptic soy agar (TSA), which was purchased from Sigma Chemical, St. Louis, Mo.

Construction of Transposon Vector, pUT-miniTn5 : *vgb* (Cm)

The 1.4 kb *Vitreoscilla* fragment containing *vgb* was removed from pUC8 : 16 by restriction with HindIII and BamHI, and inserted into the same sites in the polylinker of the pUC18 : NotI. The fragment was then re-transferred by restriction with NotI into the pUT-miniTn5 (Cm). The ligated mixture was transformed into *E. coli* SM10 (λ*pir*) and transformants bearing *vgb* were randomly selected on LB plates in the presence of ampicillin (100 μg/mL) and chloramphenicol (24 μg/mL).

Polymerase Chain Reaction

Purification of genomic DNA of the exconjugants was performed with the Wizard Genomic DNA Purification System (Promega). In a 0.5-mL thin-wall tube, the following components were combined: 2 μL of 10x PCR-reaction buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3), 10 mM of dNTP mix, 1 μg of template DNA, 10 pmol of primer 5'CATCAAAGCCACTGTTCCTGT3' (left) and 5'CTTCTTTAATCGCACCCAACA3' (right), 0.2 μL of Taq DNA polymerase (Promega), and 16.2 μL of H_2O). Amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer, Wellesley, MA) for 35 cycles, each cycle being 30 seconds at 95°C (denaturation), 30 seconds at 55°C (hybridization), and 30 seconds at 72°C (elongation).

Spectrophotometric Analysis of VHb Expression

The recombinant cells were spectrophotometrically examined for VHb expression using a Varian Cary Model 210 scanning spectrophotometer, with either whole cells or cytoplasm. For whole cell analysis, *Burkholderia* strains were incubated in TSB in shake flasks overnight at 30°C, and samples were taken from stationary phase cultures. For Cytosolic analysis of *B. cepacia*, cells were rown to stationary phase at 30°C in 1L flasks containing 500 mL of TSB. Cytosol was prepared by a modifaication of the method of Abrams and Webster[11]. The cytosolic fractions were diluted with 20 mM potassium phosphate, pH 7.2, to protein concentrations of 30 mg/mL and used directly for CO-difference spectral analysis. After harvesting by centrifugation (6,000 g for 10 minutes at 4°C), the cells were washed twice with 20 mL 100 mM sodium phosphate, pH 7.4 followed each time by a 10 minute centrifugation as described above. Then approximately 0.5 g wet weight of bacterial cell pastes was suspended in 20 mL of the same buffer. A baseline was obtained with these suspensions in both the reference and sample chambers. The sample cuvette was then bubbled with CO for 3 minutes and rescanned as previously described to give a CO-difference spectrum[12]. Data were fed into a Macintosh computer and smoothed by the Gaussian method using OCR software.

Southern Hybridization

Ten microgram of genomic DNA samples disgested with restriction enzymes, and the fragments were separated by electrophoresis through 1% agarose gels in 1X TAC buffer. 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 (*vgb*) and hybridization were performed with the ECL system (Amersham) following the manufacturer's protocol.

Growth Comparisons in Rich Medium

Once the exconjugants *Burkholderia cepacia* bearing *vgb* were identified, their growth and viability

was examined in comparison with wild type host strains. Shake flask cultures of strains (either *vgb*-free or *vgb*-bearing) to be used as inocula were grown aerobically in 25 mL of TSB for 12 hours in a Fisher model 129 shaking water bath at 30°C and 200 rpm. Then, approximately 0.1 mL of each culture (volume adjusted to give a constant A_{600} of cells) was harvested by a 5 minute centrifugation at 14,000 g in a Fisher 235B microcentrifuge, washed twice with 30°C fresh medium and inoculated into 50 or 200 mL medium as needed.

Normal oxygen condition was 200 rpm in Erlenmeyer flasks in which the medium volume was 20% of the flask volume; restricted oxygen condition 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 on TSB. Samples of cultures were taken at intervals during growth. All samples were serially diluted with 0.85% sodium chloride solution and appropriate dilutions were plated in triplicate on the plates. Colonies were counted after growth for 16 hours at 30°C. In order to investigate the viability after long periods in culture, the growth experiments in rich media lasted 144 hours. 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.

All the individual experiments were conducted under both high and low oxygen conditions described as above, and 1,000 ppm of benzoate was added to the medium as indicated.

Growth Comparison in Minimal-Benzoate Medium

The growth comparison in minimal-benzoate medium was done by the procedure described above with several exceptions. Briefly, total growth time was 72 hours instead of 144 hours, due to the limited nutrient supplied. Samples were taken every 6 hours instead of 4 hours in the log phase, also due to the slower growth rate in minimal-benzoate medium. At each time point samples were also taken, centrifuged at 14,000 g in a microcentrifuge for 5 minutes, in duplicate, for measuring the benzoic acid degradation rate by quantitating the benzoic acid remaining in the medium (Na_2HPO_4 3.0 g/L, KH_2PO_4 1.5 g/L, NH_4Cl 1.0 g/L, NaCl 0.5 g/L, $MgSO_4$ 0.24 g/L, $CaCl_2.2H_2O$ 0.015 g/L, $FeCl_3.6H_2O$ 0.05 g/L, pH 7.4) containing 1,000 ppm of Benzoic acid.

Measurement of the Ability to Degrade Benzoic Acid

The effect of *vgb*/VHb regarding degradation of benzoic acid was examined with high-performance liquid chromatography (HPLC). *B. cepacia* (with and without *vgb*) was grown at 30°C in minimal or rich medium containing benzoate at 1,000 ppm. At each time point during the growth, cells were removed by centrifugation and supernatant fluids were analyzed by HPLC (Varian Star Chromatography ISO 9001 system) using a Spherisorb C18 reverse phase column with acetonitrile and trifluoroacetic acid as the mobile phase. The elution gradient was 25% acetonitrile and 75% aqueous trifluroroacetic acid changed to 50% acetonitrile and 50% aqueous trifluoroacetic acid over 10 minutes with a flow rate of 1.5 mL/min. All compounds were detected by absorbance at 230 nm.

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