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ORIGINAL PAPER

Biodegradation of vinyl chloride, *cis*-dichloroethene and 1,2-dichloroethane in the alkene/alkane-oxidising *Mycobacterium* strain NBB4

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Abstract *Mycobacterium chubuense* strain NBB4 can grow on both alkanes and alkenes as carbon sources, and was hypothesised to be an effective bioremediation agent for chlorinated aliphatic pollutants. In this study, the ability of NBB4 to biodegrade vinyl chloride (VC), cis-dichloroethene (cDCE) and 1,2-dichloroethane (DCA) was investigated under pure-culture conditions and in microcosms. Ethene-grown NBB4 cells were capable of biodegrading VC and cDCE, while ethane-grown cells could biodegrade cDCE and DCA. The stoichiometry of inorganic chloride release (1 mol/mol in each case) indicated that VC was completely dechlorinated, while cDCE and DCA were only partially dechlorinated, yielding chloroacetate in the case of DCA, and unknown metabolites in the case of cDCE. The apparent maximum specific activities (k) of whole cells against ethene, cDCE, ethane and DCA were 93 ± 4.6, 89 ± 18, 39 ± 5.5, and 4.8 ± 0.9 nmol/min/mg protein, respectively, while the substrate affinities (K_S) of whole cells with the same substrates were 2.0 ± 0.15, 46 ± 11, 11 ± 0.33 and 4.0 ± 3.2 μM, respectively. In microcosms containing contaminated aquifer sediments and groundwater, NBB4 cells removed 85-95% of the pollutants (cDCE or DCA at 2 mM) within 24 h, and the cells remained viable for >1 month. Due to its favourable kinetic parameters, and robust survival and biodegradation activities, strain NBB4 is a promising candidate for bioremediation of chlorinated aliphatic pollutants.

Keywords *Mycobacterium* · Biodegradation · Bioremediation · Microcosm · Vinyl chloride · *Cis*-dichloroethene · 1,2-dichloroethane · Alkane · Alkene · Monooxygenase

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Introduction

Chlorinated aliphatic compounds have natural sources (Gribble 1998), but they become pollutants when they occur in the environment at high concentrations due to industrial activities. Chlorinated ethenes such as perchloroethene (PCE) and trichloroethene (TCE) have been widely used as solvents, while vinyl chloride (VC) and 1,2-dichloroethane (DCA) are used as intermediates in the production of polyvinyl chloride (PVC) plastic (Mattes et al. 2010). Due to their extensive use and improper disposal, the chlorinated ethenes and ethanes have become major contaminants of groundwater (Squillace et al. 1999), and the remediation of sites contaminated with these compounds is a priority as they are toxic and carcinogenic (Kielhorn et al. 2000). Under anaerobic conditions, chlorinated aliphatics can be dechlorinated by bacteria such as *Dehalococcoides* spp., yielding

less-chlorinated ethenes as end-products (Smidt and de Vos 2004). However, these anaerobic processes may fail to go to completion, resulting in accumulation of more-hazardous compounds such as VC. For this reason, there is great interest in investigating the potential of aerobic bacteria for bioremediation of the lesser-chlorinated ethenes and ethanes—this would allow sequential anaerobic–aerobic treatment for complete pollutant removal (Miller et al. 2007).

Aerobic biodegradation of chlorinated ethenes typically occurs due to the initial action of dioxygenase and monooxygenase (MO) enzymes. Due to their broad substrate range, oxygenases can fortuitously attack non-growth substrates in a process known as cometabolism (Wackett 1995). Cometabolism is the only aerobic mode of attack on highly-chlorinated ethenes (PCE, TCE), while the lesser-chlorinated ethenes can be attacked by both cometabolic and growth-linked processes (Oldenhuis et al. 1989; Ewers et al. 1990; Ensign et al. 1992; Hartmans and De Bont 1992; Melin et al. 1996; van Hylckama Vlieg et al. 1998; Koziollek et al. 1999; Steffan et al. 1999; Kim et al. 2000; Verce and Freedman 2000; Hage et al. 2001; Coleman et al. 2002a, b; Verce et al. 2002; Rui et al. 2004; Frascari et al. 2008; Wood 2008; Zhao et al. 2010). Aerobic biodegradation of the chlorinated ethanes can be initiated via either hydrolase attack (Janssen et al. 1985; van den Wijngaard et al. 1992) or MO attack (Oldenhuis et al. 1989; Oldenhuis et al. 1991; Chang and Alvarez-Cohen 1996; Ely et al. 1997; Yagi et al. 1999; Kim et al. 2000; Hage et al. 2001). Aerobic biodegradation of trichloroethanes occurs via cometabolism only, but the di- and monochlorinated ethanes can be metabolised via either cometabolism or growth-linked processes.

Growth-linked processes have some advantages over cometabolism for bioremediation (Alvarez-Cohen and Speitel 2001; Arp et al. 2001), but the responsible bacteria are rare, and thus bioaugmentation would be needed at most sites—this is problematic, since the added degraders are not adapted to the local conditions. In the case of DCA, bioaugmentation with lab isolates has been shown to be feasible for field-scale bioremediation (Stucki and Thuer 1995), but long lag periods (several years) were required, possibly for adaptation of the inoculant strains to the local conditions. In some cases, successful aerobic bioremediation of chlorinated ethenes and ethanes can be accomplished simply by monitored natural attenuation (Witt et al. 2002) or air-sparging (Davis et al. 2009) with no addition of hydrocarbon co-substrates or bacterial cells. Bacteria capable of cometabolism of chlorinated aliphatics are widespread in the environment, which is advantageous for bioremediation (Speitel and Closmann 1991; Fries et al. 1997; Azizian et al. 2005; Connon et al. 2005; Semprini et al. 2007). Despite their lack of growth on chlorinated aliphatics, the cometabolic degraders can have very high specific activities and high affinities with pollutants (Oldenhuis et al. 1991; Alvarez-Cohen and Speitel 2001).

Mycobacterium strain NBB4 was previously isolated from estuarine sediment using ethene as the sole carbon and energy source (Coleman et al. 2006); and identified as *M. chubuense* on the basis of 16S rDNA sequencing. Strain NBB4 contains an unprecedented diversity of soluble di-iron MO (SDIMO) genes (Coleman et al. 2006), along with three cytochrome p-450's from the CYP153 family, and an AlkB homolog (membrane-bound di-iron monooxygenase) (Genbank accession # GU174750-GU174753, GU 145557). The hydrocarbon growth substrate range of NBB4 is very broad, including ethene, propene, and both gaseous and liquid *n*-alkanes (C_2–C_{16}) (Coleman et al. 2010), suggesting that this strain may be useful for bioremediation. The aims of this study were to test the hypothesis that strain NBB4 was capable of aerobic cometabolic biodegradation of chlorinated ethenes and ethanes, to evaluate the kinetic parameters of biodegradation, and test the ability of NBB4 to biodegrade pollutants and survive in microcosms containing aquifer sediments from a DCA-contaminated site.

Materials and methods

Chemicals, bacterial strains, and growth media

Ethene was obtained from BOC gases (Australia) and was 99% pure. Ethane (99+% purity), VC (99.5+% purity), cDCE (97% purity, 3% trans-DCE, and traces of chloroform) and DCA (99.5+% purity) and all other chemicals (analytical grade) were obtained from Sigma–Aldrich Co. (Castle Hill, Sydney). Reverse osmosis (RO) water was used for preparing media and buffers. *M. chubuense* strain NBB4 was

isolated as described previously (Coleman et al. 2006). Inoculum cultures were grown in TSG medium (3 g/l trypticase soy broth powder, 10 g/l glucose), and the cells washed twice with KP buffer (20 mM K_2HPO_4, 0.02% Tween 80, pH 7.0) and frozen in small single-use aliquots in the same buffer. Cultures for experimental assays were grown in mineral salts medium (MSM) (Coleman et al. 2002a, b) containing 0.02% (v/v) Tween-80 to minimise clumping, and either ethane or ethene (10% vol/vol in air headspace) as carbon sources. Small-scale cultures were grown in 30 ml medium in 120 ml serum bottles with butyl rubber stoppers, and aluminium crimp-seals. Large-scale cultures were grown in 300 ml medium in 1.2 l Schott bottles with a custom-made gas-tight closure, consisting of a polybutylene terephthalate screw-cap with a 1 mm hole drilled in the centre, on top of a PTFE-faced silicone rubber seal (44 mm × 2 mm, Duran). Cultures were checked for purity after each experiment on TSG plates. All cultures were grown aerobically at 30°C, with broths shaken at 150 rpm.

Resting cell suspensions and protein assay

Cultures of NBB4 were inoculated at $OD_{600} = 0.1$, and grown for 40–50 h in MSM-ethene or MSM-ethane broth until the OD_{600} was between 0.6 and 0.8, corresponding to late exponential growth phase (the maximum OD_{600} obtained on these substrates under these conditions was approx. 1.2 (data not shown)). Cells were harvested by centrifugation at 6000×*g* for 10 min, washed three times in KP buffer, and resuspended in 1 ml of the same buffer. Resting cell suspensions were prepared in 16 ml serum bottles in a final volume of 4 ml KP buffer at OD_{600} values ranging from 2 to 27 depending on the prior growth substrate and type of assay being conducted (see below for details). Bottles were capped with PTFE-faced butyl rubber stoppers, and sealed with aluminium crimp-seals. Substrates were equilibrated at 30°C for 30 min in 3 ml KP buffer before assays were started by injection of NBB4 cells in a 1 ml volume of the same buffer, and the bottles incubated horizontally with shaking at 200 rpm at 30°C. Protein was quantified in cell suspensions by a UV absorbance method after lysis with alkali and heat, as described previously (Coleman et al. 2002a, b).

Gas chromatography

Quantitation of all volatile compounds (ethene, ethane, VC, cDCE, DCA) was carried out by gas chromatography (GC) using a Hewlett Packard 5890E-II machine with an HP-PLOT Q capillary column (15 m × 0.53 mm, Agilent) and Flame Ionization Detector (FID), run in splitless mode. Headspace samples (100 µl for ethene, ethane, VC and 250 µl for cDCE and DCA) were injected onto the column and analysed using a 5 min isocratic run (oven temperature 200°C, injector temperature 200°C, detector temperature 250°C, carrier flow rate: 2 ml per min). Ultra-high purity helium (99.999%, BOC gases) was used as the carrier gas, while the FID was supplied with high purity H_2 (99.99%), high purity N_2 (99.99%) and instrument grade air (99.99%) (all from BOC gases). The retention times of ethene, ethane, VC, cDCE and DCA were 1.2, 1.3, 2.1, 2.2, and 2.9 min, respectively. Analytes were quantified by reference to external standard curves constructed from at least five data points, using triplicate injections at each point. Standards were set up in identical serum bottles containing the same liquid volume (KP buffer) as test samples, and were equilibrated for at least 30 min with shaking at 30°C before analysis.

Stoichiometry of chloride release from pollutants

Resting cell suspensions were prepared at densities of $OD_{600} = 8$ (ethene-grown cells) or $OD_{600} = 27$ (ethane-grown cells). These values were chosen based on preliminary experiments to provide sufficient biomass to enable complete turnover of 10 µmol of chlorinated pollutants within a 24 h experimental time-scale. Duplicate bottles were set up for each of the following conditions; ethene-grown cells with VC, ethene-grown cells with cDCE, ethane-grown cells with DCA, ethene-grown cells with no pollutant, ethane-grown cells with no pollutant. In all cases, the initial amount of substrate added was 8 ± 1 µmol—this was added as either 200 µl of neat gas (VC) or as 320 µl of a 25 mM stock solution in KP buffer (cDCE and DCA). At zero time (cell addition), bottles were shaken vigorously by hand (10 s), and a headspace sample taken immediately from one bottle of each pair for GC analysis to determine the amount of pollutant, while the second

bottle from each pair was sacrificed to determine the initial chloride concentration via centrifuging the cells (4°C, 1 min, 16,000×*g*), filtering the supernatant (0.22 μm pore-size membrane filter) and storing the supernatant at −20°C for later analysis. The remaining bottles were incubated with shaking for 24 h, sampled again for pollutants via GC, and sacrificed for chloride assay as described above. A method based on that of Bergmann and Sanik (Bergmann and Sanik 1957) was used for quantitation of inorganic chloride, as follows. Resting cell supernatant (1 ml) was mixed with 200 μl iron reagent (8% (w/v) Ferric Ammonium Sulphate in 6 M Nitric Acid) and 400 μl Mercury Reagent (0.1% (w/v) mercuric thiocyanate in ethanol), incubated 10 min at room temperature, and the absorbance read at 460 nm. Chloride concentration was calculated via reference to a standard curve based on NaCl solutions (0, 0.1, 0.2, 0.5, 1.0 mM) prepared in KP buffer. Test samples were diluted where necessary to bring them within the absorbance range of the standard curve (A_{460} approx 0.045–1.080). The whole experiment was repeated four times, and the stoichiometry of chloride release calculated as the average number of moles Cl^- produced per mole of chlorinated substrate degraded.

Use of haloacid dehalogenase and chloride assay to detect chloroacetate

The haloacid dehalogenase gene (*dhlB*) from *Xanthobacter autotrophicus* GJ10 was amplified by PCR from plasmid pJ66 (van der Ploeg et al. 1991) using primers NVC309 (GAGGGATCCCATGATCAAGG CAGTCGTG) and NVC310 (ATCGGATCCATGC CTCGTCACAC), and the 789 bp product was cloned into the *Bam*HI site of the pET-15b vector (Novagen) then transformed by heat shock into chemically-competent cells of *E. coli* TOP10 (Invitrogen). Clones containing the correct construct were detected using colony PCR with primers VMC15 (CTCAC-TATAGGGGAATTGTGAGCGG) (binds to pET-15b) and NVC310 (binds to *dhlB*), and one positive clone was retained for further work. The correct structure of the pET15b-*dhlB* construct was confirmed by restriction digest of purified plasmid using *Bam*HI, and the plasmid was transformed into the expression host *E. coli* BL21(DE3) (Invitrogen). The *E. coli* BL21(DE3)(pET15b-dhlB) strain was grown in 50 ml LB broth with ampicillin (100 μg/ml) to mid-exponential phase ($OD_{600} = 0.5$) and expression of dhlB was induced with 1 mM IPTG for 2.5 h. Cells were washed 3 times in KP buffer, then suspended at an OD_{600} of 20 ± 2 in 500 μl cell suspensions containing either KP buffer, 2 mM chloroacetate, or supernatants from NBB4 cometabolism experiments (cDCE/DCA), and incubated at 37°C with shaking at 200 rpm for 16 h. The above was repeated for BL21 cells transformed with vector only as a control. Cells were removed by centrifugation (16,000×*g*, 5 min), and the supernatant from each cell suspension was tested for inorganic chloride as described above.

Kinetic studies of hydrocarbon and chlorinated hydrocarbon biodegradation

Resting cell suspensions were prepared at densities of $OD_{600} = 2$ (ethene-grown cells) or $OD_{600} = 15$ (ethane-grown cells), and substrates added at 4 μmol per bottle. Bottles were shaken as described above, with headspace samples taken for GC analysis approximately every 10 min until the substrate had been depleted to below detection limits (between 2 and 7 h, depending on substrate). This experiment was repeated at least three times for each compound. Substrate depletion curves were plotted and the apparent maximum specific activity of whole cells (*k*, nmol substrate consumed/min/mg protein) was calculated from the initial straight-line portion of the graph. The apparent substrate affinity of whole cells (K_S, μM) was calculated using the integrated form of the Michaelis–Menten equation (Stoida and Emelyanov 1980) using a subset of the substrate depletion data around the point where the reaction rate became non-linear. Specifically, the approach used was to plot $(S_0 - S_t)/t$ against $(2.303/t \times \ln (S_0/S_t))$, where t = time, S_0 = initial substrate concentration, and S_t = substrate concentration at time "t". Assuming the reaction obeys Michaelis–Menten kinetics, then this plot will be linear, with a slope = $1/K_S$. Average k and K_S values were derived from at least three independent experiments. The aqueous concentrations of substrates were calculated from the total amounts in the test bottles using Henry's law (Gossett 1987), and the appropriate dimensionless Henry's constants at 30°C, which were 9.09 for ethene (Kruis and May 1962), 5.29 for ethane (Sander 1999), 1.27

for VC (Gossett 1987), 0.19 for cDCE (Gossett 1987) and 1.26 for DCA (Gossett 1987).

Site description and sample collection

For the construction of microcosms (section below), contaminated site material was collected on 22 February, 2008 at the Orica Southlands site, in the Botany Industrial Park (Sydney, Australia). This site has a long history of contamination by chlorinated pollutants, including DCA, chlorinated ethenes, hexachlorobenzene, and hexachlorobutadiene, and is in the process of being remediated via a variety of methods (http://www.oricabotanytransformation.com/). The main contaminant of concern is DCA, which is present as a dense non-aqueous phase liquid (DNAPL) in several subsurface locations, leading to concentrations of DCA in groundwater as high as 6 g/l near the source area. Details of the physico-chemical and hydrogeological properties of this contaminant plume and the surrounding Botany Sands aquifer have been published previously (Jankowski and Beck 2000; Acworth 2001). For the present study, aquifer sediment and groundwater samples were collected from 6 m below ground surface, immediately upgradient of the Pilot Scale Reactive Iron Barrier (PSRIB) located on Southlands Block 1. (latitude −33.95596, longitude 151.21652). Sediments were obtained via a core-sample drill, while groundwater was obtained by peristaltic pumping from an adjacent monitoring well.(WG136). Details of the groundwater chemistry at the sampling site were as follows: pH, 6.45; redox potential (E_h), 185 mV; dissolved oxygen, 0.75 mg/l; temperature, 22.1°C; dissolved organic carbon, 92 mg/l; electrical conductivity, 2305 µS/cm. The detectable contaminants in the groundwater at the sampling site were as follows (all in mg/l): VC, 11.7; DCA, 6.04; 1,1-dichloroethane, 0.066; cDCE, 0.034; dichloromethane, 0.026; 1,1,2-trichloroethane, 0.011.

Microcosm study of bioaugmentation

Microcosms were set up in 16 ml serum bottles, using a mixture of aquifer sediment (2 g wet weight) and groundwater (3 ml) to give a total volume of 3.8 ml site material. The site materials were used without any nutrient amendments or pH adjustment. The bottles were sealed with PTFE-faced butyl rubber stoppers and crimp-sealed with aluminium caps. Since the aquifer sediments we were able to access were from a less-polluted part of the site, and the traces of volatile pollutants described in analytical samples (above) were mostly lost during sampling, transport, and microcosm setup, we amended the microcosms with chlorinated pollutants (either DCA or cDCE) to a final concentration of 2 mM. To determine whether a growth substrate was simultaneously required for chlorinated pollutant degradation, in some experiments, ethane or ethene were injected into DCA or cDCE microcosms, respectively, at 1% of the headspace volume. Prior to starting experiments by addition of cells, microcosms were equilibrated at 30°C for 30 min, with bottles laid horizontally, and shaking at 200 rpm. Cells of NBB4 grown on ethene or ethane, and washed three times in KP buffer (as above) were washed one further time in water, resuspended in water, and 0.2 ml was injected into cDCE and DCA microcosms to give a predicted final OD_{600} of 0.8 and 27, respectively (note that these OD_{600} values refer to cells only, not aquifer materials, and are predicted values based on the absorbance of the inoculum cell suspensions). The rationale for using higher cell densities of ethane-grown cells than ethene-grown cells was the much lower specific activity of the former, especially on organochlorine substrates (see Tables 1 and 2) Immediately following injection of cells, the bottles were shaken vigorously by hand for 10 s, a "zero time" headspace sample was taken for GC analysis, and the bottles were returned to the shaker. Headspace sampling continued over a 24 h period to monitor substrate disappearance. Controls included uninoculated bottles (to investigate the bioremediation potential of the native site microflora), and bottles containing autoclaved site materials (to investigate abiotic losses of pollutant). Microcosms were set up in triplicate for each study condition.

Table 1 Apparent specific activities (k) and affinity constants (K_s) in pure cultures

Assay substrate	Growth substrate	k (nmol/min/mg protein) (n)	K_s (µM) (n)
Ethene	Ethene	93 ± 4.6 (3)	2.0 ± 0.15 (3)
cDCE	Ethene	89 ± 18 (3)	46 ± 11 (3)
Ethane	Ethane	39 ± 5.5 (3)	11 ± 0.33 (3)
DCA	Ethane	4.8 ± 0.9 (6)	4.0 ± 3.2 (6)

Table 2 Apparent specific activities (k) determined in microcosms

Assay substrate	Competing substrate	Growth substrate	k (nmol/min/mg protein) (n)
cDCE	None	Ethene	49 ± 18 (12)
cDCE	Ethene	Ethene	48 ± 16 (12)
Ethene	cDCE	Ethene	14 ± 5.8 (12)
DCA	None	Ethane	5.0 ± 1.9 (3)
DCA	Ethane	Ethane	5.2 ± 2.2 (3)
Ethane	DCA	Ethane	0.15 ± 0.02 (3)

Total and viable cell counts

Total counts of bacteria in the aquifer sediment/groundwater slurry used in experimental microcosms were carried out by fluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) staining (Janssen et al. 2002). Thirty fields of view were counted, and the results were averaged to obtain the total microscopic cell count for the sample. Viable counts of aerobic heterotrophic bacteria in the same samples were done via spread-plating dilutions onto triplicate R2A® plates (Oxoid), with incubation for 1 week at 30°C before counting colonies. The average count was obtained from a set of three plates at one dilution showing between 30 and 300 colonies. Viable counts were also done from inoculated microcosms at various intervals (0, 1, 7, 30 days) to determine the survival of NBB4 cells under the microcosm conditions. Although no specific selective marker was carried by the NBB4 cells, their very high numbers relative to other microflora in the samples (2–3 orders of magnitude higher), their distinctive morphology (bright yellow, opaque, mucoid colonies—these types were not seen in native site samples) and their distinctive pattern of appearance (small colonies appearing on plates after 1 week) enabled the NBB4 cells to be easily counted on R2A plates against the background of site microflora.

Results

Strain *NBB4* can dechlorinate VC, cDCE and DCA

The unusual ability of *M. chubuense* strain NBB4 to grow on both alkenes and alkanes as carbon sources suggested that this bacterium might be uniquely useful for bioremediation of sites contaminated with both chloroethene and chloroethane pollutants. We examined this via GC analysis of pollutant depletion and a colorimetric assay to detect production of inorganic chloride. Preliminary experiments (data not shown) indicated that NBB4 could not grow on chlorinated compounds (VC, cDCE, TCE, DCA) as sole carbon sources, cells grown in non-hydrocarbon media (TSG or MSM-acetate) did not attack chlorinated compounds, ethene-grown cells degraded VC and cDCE, but not DCA or TCE, and ethane-grown cells degraded cDCE and DCA, but not TCE. Since ethene-grown cells had higher activity against chloroethenes than ethane-grown cells, in all further experiments, ethene-grown cells were used for testing biodegradation of VC and cDCE, while ethane-grown cells were used for testing biodegradation of DCA.

Resting cell experiments showed that after 24 h incubation, ethene-grown cells consumed all of the VC or cDCE provided in the assay (approx 8 μmol, corresponding to approx. 2 mM initial concentration), and ethane-grown cells consumed all the DCA (8 μmol = 2 mM), allowing us to determine the stoichiometry of chloride release from these substrates, which was 0.88 (± 0.01) mol/mol for VC, 1.17 (± 0.27) mol/mol for cDCE, and 1.06 (± 0.16) mol/mol for DCA. No chloride production was observed in controls consisting of live cells with no substrate added, or killed cells with substrate added. Since the VC molecule has a single chlorine atom, while cDCE and DCA have two chlorine atoms, the data indicate that VC was completely dechlorinated, while cDCE and DCA were only 50% dechlorinated, and thus, organochlorine metabolites were most likely accumulating in reactions in the latter two cases.

Based on previous work (McCall et al. 1983; Fox et al. 1990; Hage and Hartmans 1999), we hypothesised that chloroacetate was a likely metabolite of cometabolic cDCE and DCA oxidation in NBB4 cells, and searched for evidence of this metabolite in resting cell suspension supernatants by adding *E. coli* cells overexpressing a haloacid dehalogenase enzyme, then performing chloride analysis (Fig. 1). Confirmation that the haloacid dehalogenase was actively expressed and that the experimental strategy was valid were obtained by the observation that 2 mM chloride was released from a 2 mM chloroacetate solution upon

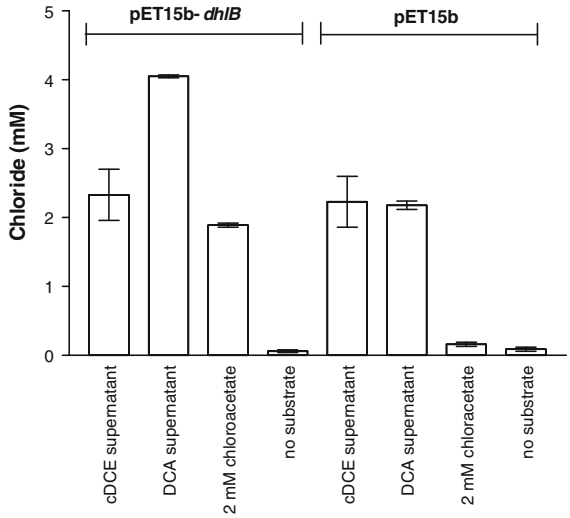

Fig. 1 Chloride release from NBB4 resting cell supernatants and chloroacetate standards after addition of *E. coli* BL21(DE3) cells containing either pET15b or pET15b-*dhlB*. Results represent averages ± standard deviations ($n = 3$)

addition of *E. coli* BL21(DE3)(pET15b) cells, but no chloride was released when cells carrying vector alone were added. Addition of *E. coli* cells carrying the vector alone to resting cell supernatants did not significantly change the chloride concentrations observed in earlier experiments. Addition of *E. coli* cells expressing DhlB did not change the chloride concentration in supernatants from cDCE cometabolism experiments, indicating that chloroacetate was not produced as a metabolite of cDCE oxidation by NBB4. Addition of *E. coli* cells overexpressing DhlB to supernatants from DCA cometabolism experiments resulted in a doubling of the chloride concentration relative to incubations with cells carrying vector only, from 2.18 ± 0.06 mM to 4.05 ± 0.02 mM—this represents complete recovery of all chlorine from DCA as inorganic chloride, and indicates that NBB4 cells oxidise DCA solely to chloroacetate, with negligible amounts of other organochlorine metabolites produced.

Cells of NBB4 have high specific activity and high affinity for chlorinated pollutants

To qualify as a useful agent of bioremediation, a microorganism should be able to metabolise the pollutant of interest at a high rate and with high affinity, to enable bioremediation to occur in a reasonable time-frame, and to reduce the pollutant concentration to below legally-permissible limits. With this in mind, we investigated the kinetic parameters of DCA and cDCE biodegradation using resting cells of NBB4, and compared these values to those of the natural growth substrates of the bacterium (ethene and ethane). Substrate depletion curves were obtained for ethene, ethane, cDCE and DCA (Fig. 2), and the specific activity (k) and substrate affinity (K_S) were estimated from these curves (Table 1). Note that the different initial aqueous substrate concentrations in these assays are due to the very different partitioning behaviour of the chlorinated vs. non-chlorinated substrates, and that each bottle contained the same total mass of substrate (4 μmol). Although ideally such assays would all be done at identical cell densities, because the ethene-grown cells had much higher activity than ethane-grown cells (especially on chlorinated substrates), we used higher densities of ethane-grown cells to compensate for this, such that assays could be performed over similar time-frames. With the alkenes (ethene/cDCE), we found that the specific activities of cells toward both substrates were almost identical, although the affinity of the cells for the natural substrate was 23-fold higher than for the dichlorinated xenobiotic. The kinetic parameters for the alkanes (ethane/DCA) showed a very different pattern, with cells showing an eightfold higher specific activity with ethane compared to DCA, but possessing a twofold higher affinity for DCA than for their natural substrate. The specific activities of the cells against alkenes were higher than those measured with similarly-substituted alkanes. The substrate affinities of the cells were higher with alkenes than alkanes for the natural substrates, but this pattern was reversed for the chlorinated substrates.

Bioaugmentation of microcosms with NBB4 leads to removal of cDCE and DCA

The effectiveness of NBB4 cells for biodegrading chlorinated pollutants under pure-culture conditions indicated that this bacterium was a good candidate for bioremediation. To investigate this possibility, microcosms containing aquifer sediments and groundwater from a site contaminated with chlorinated ethenes and ethanes were spiked with additional cDCE or DCA and inoculated with NBB4

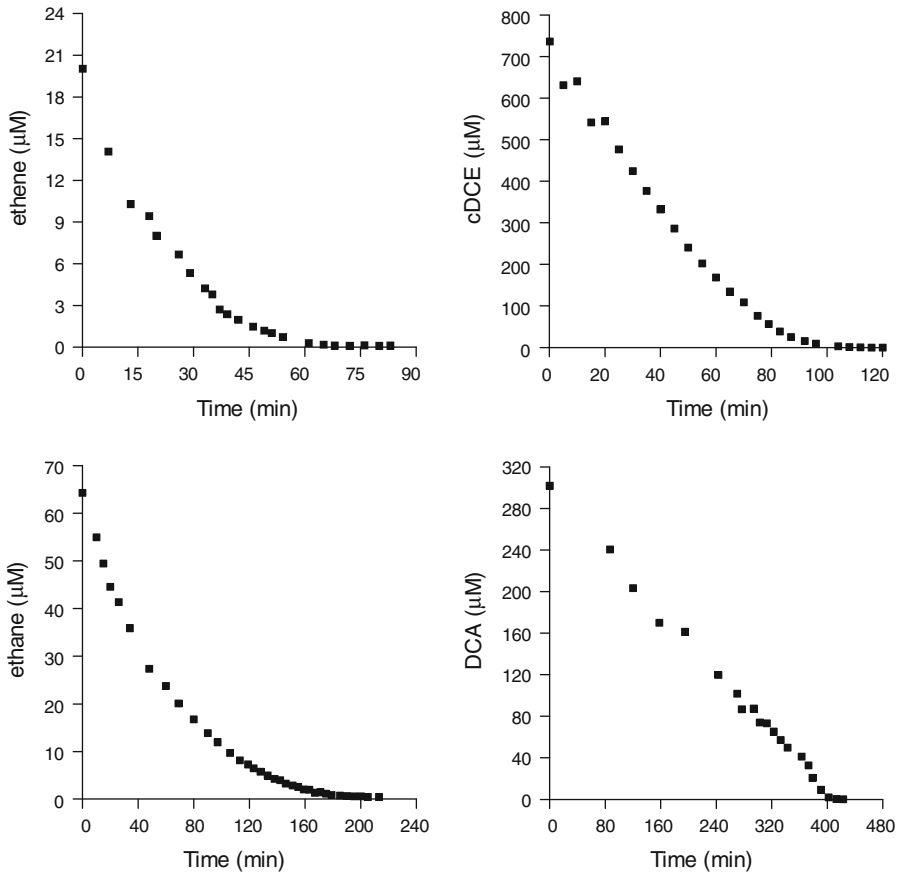

Fig. 2 Representative substrate depletion curves used for calculating K_S values in pure culture experiments. Note that three such curves (per substrate) were used for calculating the K_S values that appear in Tables 1 and 2

cells, using ethene-grown cells in cDCE microcosms and ethane-grown cells in DCA microcosms. The effect of growth substrates on pollutant degradation was investigated by the addition of ethene or ethane to one set of microcosms.

Only microcosms bioaugmented with NBB4 showed effective degradation of cDCE (Fig. 3a) and DCA (Fig. 4a). No significant loss of pollutants was observed in uninoculated microcosms or autoclaved microcosms, proving that the pollutant removal was due to the NBB4 cells, and not due to abiotic losses or to the activities of indigenous microorganisms. At the conclusion of the experiment (22 h), removal of cDCE and DCA ranged from 85 to 98% in the inoculated microcosms. The addition of ethene and ethane was not required for effective pollutant biodegradation in microcosms; the presence of ethene slowed the initial rate of cDCE biodegradation (Fig. 3a) and the presence of ethane did not affect the rate of DCA biodegradation (Fig. 4a). Both ethene (Fig. 3b) and ethane (Fig. 4b) were metabolised in microcosms containing organochlorines, although DCA appeared to have an inhibitory effect on ethane metabolism. The addition of hydrocarbon growth substrates did not dramatically alter the final extent of pollutant removal—a small increase (approx. 10%) in the amount of cDCE removed was seen in microcosms with ethene added, but this difference is within the error bars at this time point. The apparent specific activities for biodegradation of cDCE and DCA in microcosms based on the initial rate of pollutant removal were calculated to be 49 and 5 nmol/min/mg protein, respectively, which represent 55 and 105% of the activities calculated in pure culture, respectively (Table 2). The apparent specific activities of the cells in microcosms for biodegradation of ethene in the presence of cDCE, and ethane in the presence of DCA were 14 nmol/min/mg protein and 0.15 nmol/min/mg protein (Table 2), respectively, which are 15 and 0.4%, respectively, of the value obtained for metabolism of these hydrocarbons in pure culture in the absence of chlorinated pollutants (Table 1).

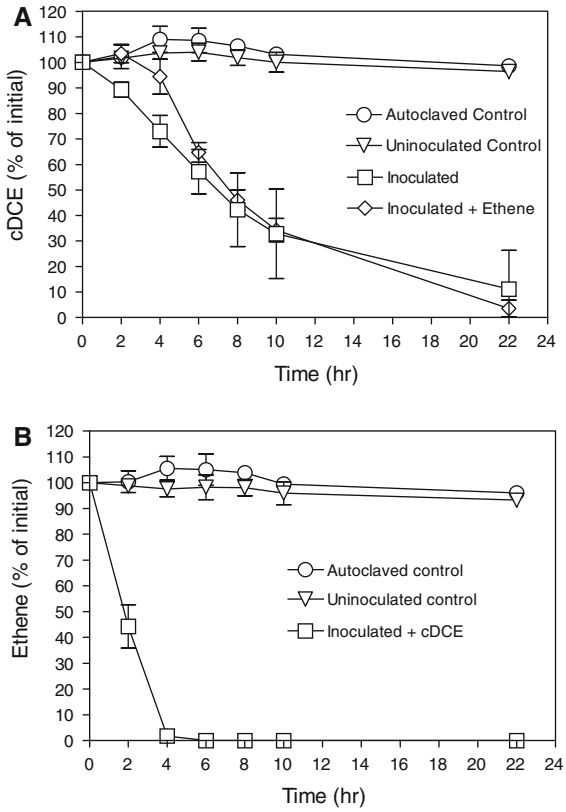

Fig. 3 **a** Biodegradation of cDCE in microcosms inoculated with ethene-grown NBB4 cells. The initial amounts of cDCE ranged between 5.25 and 6.85 μmol (approx 2 mM). **b** Metabolism of ethene in microcosms containing cDCE. The initial amounts of ethene ranged between 8.2 and 9.6 μmol (approx 1% of headspace volume). In both **a** and **b**, data points are averages of three independent experiments, *error bars* show standard deviations

Viable cells of NBB4 persist in microcosms containing cDCE and DCA

Before setup of microcosms, the numbers of indigenous bacteria in the contaminated site material (a slurry of aquifer sediment and groundwater) were estimated by two methods—DAPI staining with fluorescence microscopy, and dilution plating on R2A agar. The microscopic count was 2.04×10^8 cells/ml, while the viable count of aerobic heterotrophs was 4.40×10^6 CFU/ml. These numbers are typical for a shallow subsurface aquifer (Bone and Balkwill 1988) and indicate that approx 2% of the microscopically-visible cells were culturable under these conditions, which is within the expected range for environmental bacteria (Janssen et al. 2002). One important reason for determining the numbers of culturable aerobic heterotrophs in the site samples was that this result would influence the methods used for enumerating NBB4 cells. Since the counts of indigenous microflora were approx. 100-fold lower than the inoculated cell numbers of NBB4, and since NBB4 cells had a unique colony morphology and pattern of appearance on plates, we were able to use a simple plating assay on R2A to monitor the viability of NBB4 in the microcosms, without the need to tag the inoculum cells with a selective marker.

In the cDCE microcosms (initially inoculated at 2×10^8 NBB4 cells/ml), the viable count of NBB4 declined by approx 10- to 50-fold over the first week, but then stabilised to give counts of approx 10^7 cells/ml at 1 month after inoculation (Fig. 5a). The presence of cDCE or cDCE and ethene together in the microcosms did not have any notable effect on the survival of NBB4 compared to microcosms with no amendments. In the DCA microcosms (initially inoculated at 5×10^9 NBB4 cells/ml), the viable count decreased more rapidly than in the cDCE microcosms, and continued to decline over the whole course of the experiment, giving counts of between 10^4 and 10^5 NBB4 cells/ml after 1 month (Fig. 5b). In the DCA microcosms, the addition of DCA or DCA and ethane seemed to enhance survival relative to the microcosms with no amendments, although this effect was not dramatic. The poorer survival of the NBB4 cells in the DCA microcosms compared to the cDCE microcosms may be due to depletion of oxygen, since we noticed that after 1 week incubation, the aquifer solids in the DCA microcosms changed colour from light brown to black, and the sampled liquids smelled of hydrogen sulphide. Neither of these effects was noted in the cDCE microcosms, which were inoculated with approx 20-fold less cells.

Discussion

This is the first study to investigate the ability of a combined alkene/alkane-assimilating bacterium to biodegrade chlorinated aliphatic pollutants, and we have demonstrated that *M. chubuense* strain NBB4 is very effective for this application. The bacteria attack cDCE and DCA at high rates and with high affinity, and remove these pollutants to below detection limits

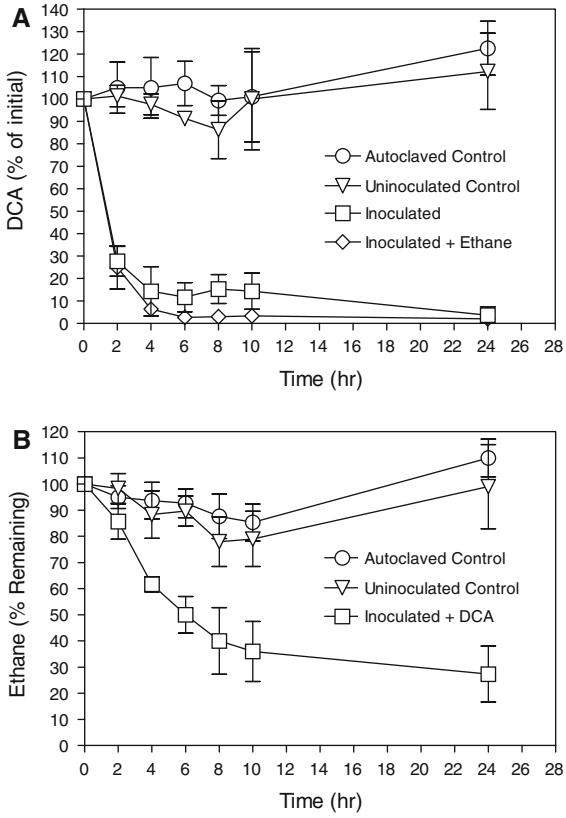

Fig. 4 **a** Biodegradation of DCA in microcosms inoculated with ethane-grown NBB4 cells. The initial amounts of DCA ranged between 6.39 and 8.58 μmol (approx 2 mM). **b** Metabolism of ethane in microcosms containing DCA. The initial amounts of ethane ranged between 8.3 and 9.6 μmol (approx 1% of headspace volume). In both **a** and **b**, data points are averages of three independent experiments, *error bars* show standard deviations

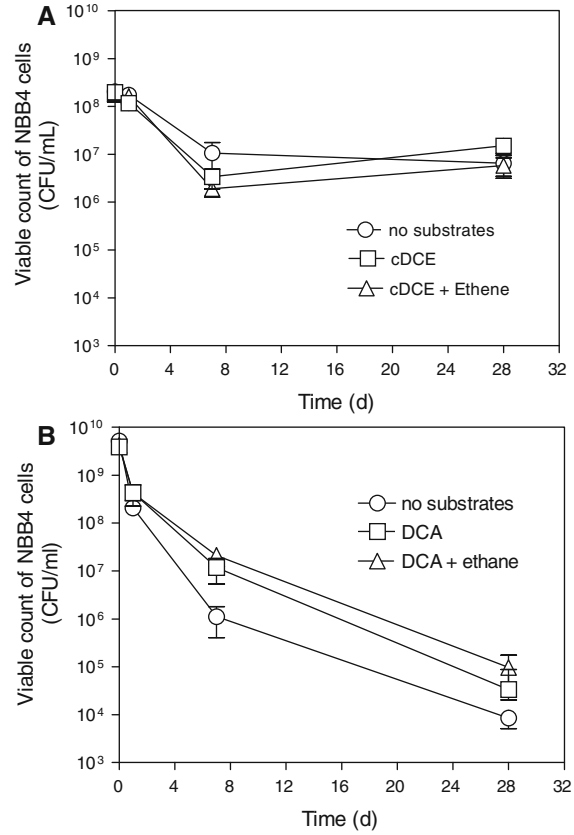

Fig. 5 Survival of *Mycobacterium* strain NBB4 cells in microcosms, as determined by dilution plating onto R2A medium. Data points are averages of three independent experiments, and *error bars* represent standard deviations ($n = 3$). **a** cDCE microcosms **b** DCA microcosms

(0.06 and 1.6 μM, respectively) in pure-culture assays. The maximum allowable concentrations of cDCE and DCA in drinking water are 0.06 mg/l (0.62 μM) and 0.003 mg/l (0.03 μM), respectively (Bursill et al. 2004), so the bacteria are clearly capable or remediating cDCE to below legal limits, although more-sensitive experimental methods would be required to determine the same for DCA. Cells of NBB4 were effective for bioremediation of contaminated site materials in microcosms—they removed nearly all of the cDCE and DCA added to the microcosms, and persisted as viable cells for at least 1 month under these conditions. Due to its ability to oxidize both chloroalkenes and chloroalkanes, we propose that strain NBB4 would be particularly useful as an agent of bioremediation at sites contaminated with mixtures of chlorinated aliphatic pollutants.

Resting cell suspensions of NBB4 were able to rapidly dechlorinate VC, cDCE and DCA, but the stoichiometry of chloride release indicated that cDCE and DCA were not completely mineralized. Chloroacetate was identified as the sole organochlorine metabolite produced from DCA, Chloroacetate has previously been seen as an intermediate in the growth-linked pathways of DCA oxidation in bacteria that use a hydrolase to attack DCA (Janssen et al. 1985; van den Wijngaard et al. 1992) and in bacteria that use a monooxygenase to attack DCA (Oldenhuis et al. 1989; Oldenhuis et al. 1991; Chang and Alvarez-Cohen 1996; Ely et al. 1997; Yagi et al. 1999; Kim et al. 2000; Hage et al. 2001). Our study is the first to identify a metabolite produced from DCA

by an ethane-oxidising bacterium. The results suggest that cometabolic DCA degraders could be converted to growth-linked DCA degraders by acquisition of a single enzyme (haloacid dehalogenase), assuming that DCA can induce the enzymes needed for its own degradation, and that the host bacterium can grow on the product of chloroacetate dehalogenation (glycolate). We showed that chloroacetate was not a metabolite of cDCE oxidation in NBB4; the identity of the organochlorine metabolite(s) produced from cDCE remains unknown.

Although the accumulation of chloroacetate (from DCA) and unknown organochlorines (from cDCE) as biodegradation end-products is of concern for the ultimate safety of bioremediation, the presence of these accumulated organochlorines did not seem to adversely affect the biodegradative activity or survival of the NBB4 cells, since substrate depletion curves gave a good fit to the expected Michaelis–Menten kinetics, and the cells survived for long periods in microcosms presumably containing these metabolites. Future work will employ the use of GC–MS and LC–MS to conclusively identify which metabolites are being formed from cDCE, and investigate the possibility of either building bacterial consortia or recombinant strains to metabolise these compounds further into harmless end-products.

Studying the kinetics of biodegradation of chlorinated vs. non-chlorinated alkenes and alkanes in NBB4 yielded important insights into the enzymes involved, and some surprising conclusions concerning the preferred substrates. The very different rates and affinities with alkenes vs. alkanes indicates that two different enzymes are most likely involved in these processes (one ethene-inducible, one ethane-inducible), which agrees with the finding that ethene-grown cells have a different cometabolic substrate range (VC, cDCE) to ethane-grown cells (cDCE and DCA). Ethene-induced cells had almost identical specific activities (approx 90 nmol/min/mg protein) towards ethene and cDCE, which was unexpected, since the literature suggests that cometabolic substrates are typically metabolised more slowly than the natural substrate (van Hylckama Vlieg et al. 1998; Koziollek et al. 1999; Yagi et al. 1999; Kim et al. 2000; Verce et al. 2002; Louarn et al. 2006). However, it is not correct to say that cDCE and ethene were equally good substrates, since the K_S values indicate a much higher affinity for ethene. The apparent maximum specific activity of NBB4 cells towards cDCE was 5–10 times higher than values previously reported for other ethene-oxidizers (van Ginkel and de Bont 1986; Verce et al. 2000; Coleman et al. 2002a, b), but this result must be interpreted with caution since experiments using volatile substrates are subject to mass-transfer effects, and the calculated specific activities are greatly dependent on the relative concentrations of substrates and cells used in individual experiments.

The kinetics of alkane biodegradation tell a very different story to that described above for alkene biodegradation, in that the apparent maximum specific activity for ethane was much higher than for DCA, but the cells had a higher affinity for DCA than ethane. The high affinity of the cells for DCA is promising for bioremediation applications, but made calculation of the Ks value challenging since the depletion curves remained linear almost all the way down to the GC detection limit (this is reflected in the large relative standard deviation of this data). The K_S value reported here for NBB4 is in the same range as that reported for *Pseudomonas* DCA1 (Hage and Hartmans 1999), suggesting that the enzymology in these two strains may be similar. At present the basis of DCA degradation in DCA1 is unknown, although some type of monooxygenase is implicated. In strain NBB4, we have recently obtained evidence that a copper monooxygenase similar to pMMO is responsible for C2–C4 alkane degradation (specifically, when copper chelators such as allylthiourea or nitrapyrin are added to the medium, growth on gaseous alkanes is inhibited, while growth on ethene and acetate is unaffected (data not shown)). Although NBB4 has lower specific activity with DCA than the best-characterised DCA-degrading bacterium (*Xanthobacter autotrophicus* GJ10, 85 nmol/min/mg protein) (Hartmans et al. 1992), it has much higher affinity for the substrate than GJ10 (4 vs. 650 μM), which would be expected to lead to more-complete removal of DCA by NBB4 during bioremediation.

The Botany Industrial Park site in south Sydney is heavily contaminated with organochlorines, especially DCA. Previous bioremediation trials at this site used an anaerobic biostimulation strategy to dechlorinate DCA (http://www.oricabotanytransformation.com/), but this approach was only partly successful, leading us to propose that aerobes might be a useful alternative for bioremediation at this site and similar

locations. Although the Botany contaminant plume would be described as "anoxic" (1–3 mg/l dissolved O_2), recent research has shown that aerobic degraders of chlorinated aliphatic pollutants remain active at very low dissolved O_2 levels e.g. as low as 0.02 mg/l (Gossett 2010). The microcosm data obtained in the present study support the application of strain NBB4 for bioremediation at sites such as Botany since the bacteria successfully removed approx. 90% of the pollutants, they did not require the presence of co-substrates to sustain enzyme induction (at least over the 24 h period studied), no chemical modifications of the site material were required, and the bacteria survived for prolonged periods in the site material, despite the initial presence of high concentrations of DCA or cDCE (2 mM). The usefulness of strain NBB4 for cDCE bioremediation appears to exceed that of physiologically-similar cometabolic cultures, e.g. *Mycobacterium* strain TRW-2 (Fathepure et al. 2005), the mixed culture K20 (Koziollek et al. 1999) and a VC-degrading enrichment (Freedman et al. 2001), which all required their growth substrate to be present for effective cDCE turnover.

Some interesting conclusions can be drawn from the behaviour of NBB4 towards the two test pollutants in pure culture vs. in microcosms. The pure-culture kinetics experiments predict that when mixtures of both natural substrate and chlorinated substrate are provided, the cells would preferentially attack ethene rather than cDCE, and DCA rather than ethane. This prediction was validated in the ethene microcosm experiments, where ethene addition slowed the initial rate of cDCE degradation, but ethane addition did not affect the initial rate of DCA degradation. This is the first study to the best of our knowledge that has demonstrated that an ethane-assimilating bacterium can effectively biodegrade 1,2-DCA. Biodegradation of 1,1,1,-trichloroethane (1,1,1,-TCA) using an ethane-oxidising *Mycobacterium* strain has been reported, but the physiology of this bacterium appears quite different to NBB4, as in this case, competitive inhibition was observed between ethane and 1,1,1-TCA (Yagi et al. 1999). Other studies have looked at cometabolism of tri- and di-chlorinated ethanes by methane oxidisers (Oldenhuis et al. 1989; Chang and Alvarez-Cohen 1996; van Hylckama Vlieg et al. 1996), propane-oxidisers (Frascari et al. 2008), butane-oxidisers (Kim et al. 2000; Jitnuyanont et al. 2001) and ammonia-oxidizers (Ely et al. 1997; Kocamemi and Cecen 2010). These studies indicate that in some cases, the natural substrate competitively inhibits chlorinated ethane biodegradation, while in other cases it does not—this likely reflects the diversity of alkane oxidation enzymes found in these bacteria.

While this study provides good proof-of-concept for the aerobic cometabolism strategy for bioremediation of chlorinated aliphatics, some caveats must be noted, as follows. The cell density used in microcosms was high (up to 5×10^9 cells/ml), and the microcosms were continuously agitated, and incubated at the optimum growth temperature of the inoculum bacteria (30°C)—many of these parameters are not representative of in situ bioremediation, but could be maintained for larger-scale ex-situ bioremediation e.g. in a bioreactor. Biodegradative activity in microcosms was only monitored for a relatively short period of time by field-trial standards (24 h), and it is not clear whether the good activity of the inoculum cells would be maintained over longer periods. The next logical steps in testing the bioremediation potential of strain NBB4 would be to investigate biodegradation over longer time-scales in larger-scale static microcosms, incubated under in situ temperatures and O_2 concentrations, and inoculated with smaller numbers of degradative cells.

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