*厌氧生物降解的冠军菌株 Dehalococcoides mccartyi strain 11a**

*Note: strain 11a and GEO are interchangeable and they may have similar function.

生物修复是迄今为止最佳的处理含有氯乙烯类污染物的工业废水，修复土壤的可持续性方案。很多人难以捕捉到这项技术的核心。成功的关键不是筛选菌株，而是找到好的被污染过的地方。那里的污泥一定“藏龙卧虎”。那里的污泥中的微生物就是提纯 Dehalococcoides 的好地方。纯菌株 *Dehalococcoides mccartyi strain 11a* 对氯乙烯类有机溶剂和二氯乙烷展现出了高效快速的降解特性，被认为是生物修复含有氯乙烯类有机溶剂污染环境的最有潜力的冠军菌株。下文即将讲述它的降解技能和提纯经历，以供同行参考。

Dehalococcoides	
Scientific classification	
Domain:	Bacteria
Phylum:	Chloroflexi
Class:	Dehalococcoidia
Genus:	*Dehalococcoides* Maymo-Gatell *et al.* 1997
Species	
• *D. mccartyi*	

Dehalococcoides is a genus of bacteria within class Dehalococcoidia that obtain energy via the oxidation of hydrogen and subsequent reductive dehalogenation of halogenated organic compounds in a mode of anaerobic respiration called organohalide respiration.[1] They are well known for their great potential to remediate halogenated ethenes and aromatics. They are the only bacteria known to transform highly chlorinated dioxins, PCBs. In addition, they are the only known bacteria to transform tetrachloroethene (perchloroethene, PCE) to ethene.

Contents

Microbiology

The first member of the genus *Dehalococcoides* was described in 1997 as *Dehalococcoides ethenogenes* strain 195. Additional *Dehalococcoides* members were later described as strains CBDB1,[2] BAV1, FL2, VS, and GT. In 2012 all yet-isolated *Dehalococcoides*strains were summarized under the new taxonomic name *D. mccartyi*.[3]

Activities

Dehalococcoides are obligately organohalide-respiring bacteria,[3] meaning that they can only grow by using halogenated compounds as electron acceptors. Currently, hydrogen (H_2) is often regarded as the only known electron donor to support growth of *dehalococcoides*bacteria.[4][5][6] However, studies have shown that utilizing various electron donors such as formate,[7] and methyl viologen,[5] have also been effective in promoting growth for various species of *dehalococcoides*. In order to perform reductive dehalogenation processes, electrons are transferred from electron donors through dehydrogenases, and ultimately utilized to reduce halogenated compounds,[3]many of which are human-synthesized chemicals acting as pollutants.[8] Furthermore, it has been shown that a majority of reductive dehalogenase activities lie within the extracellular and membranous components of *D. ethenogenes*, indicating that dechlorinationprocesses may function semi-independently from intracellular systems.[5] Currently, all known *dehalococcoides* strains require acetate for producing cellular material, however, the underlying mechanisms are not well understood as they appear to lack fundamental enzymes that complete biosynthesis cycles found in other organisms.[6]

Dehalococcoides can transform many highly toxic and/or persistent compounds. This includes tetrachloroethene (PCE) and trichloroethene (TCE) which are transformed to non-toxic ethene, and chlorinated dioxins, vinyl chloride, benzenes, polychlorinated biphenyls (PCBs), phenols and many other aromatic contaminants.[9][10][11]

Applications

Dehalococcoides can uniquely transform many highly toxic and/or persistent compounds that are not transformed by any other known bacteria, in addition to halogenated compounds that other common organohalide respirers utilize.[8][12] For example, common compounds such as chlorinated dioxins, benzenes, PCBs, phenols and many other aromatic substrates can be reduced into less harmful chemical forms.[8] However, *dehalococcoides* are currently the only known dechlorinating bacteria with the unique ability to degrade the highly recalcitrant, tetrachloroethene (PCE) and tricholoroethene (TCE) compounds into less-toxic forms that are more suitable for environmental conditions, and thus utilized

in bioremediation.[8][13][7] Their capacity to grow by using contaminants allows them to proliferate in contaminated soil or groundwater, offering promise for *in situ* decontamination efforts.

The process of transforming halogenated pollutants to non-toxic compounds involves different reductive enzymes. *D. mccartyi* strain BAV1 is able to reduce vinyl chloride, a toxic contaminant that usually originates from landfills, to ethene by using a special vinyl chloride reductase thought to be coded for by the *bvcA* gene.[14] A chlorobenzene reductive dehalogenase has also been identified in the strain CBDB1.[15]

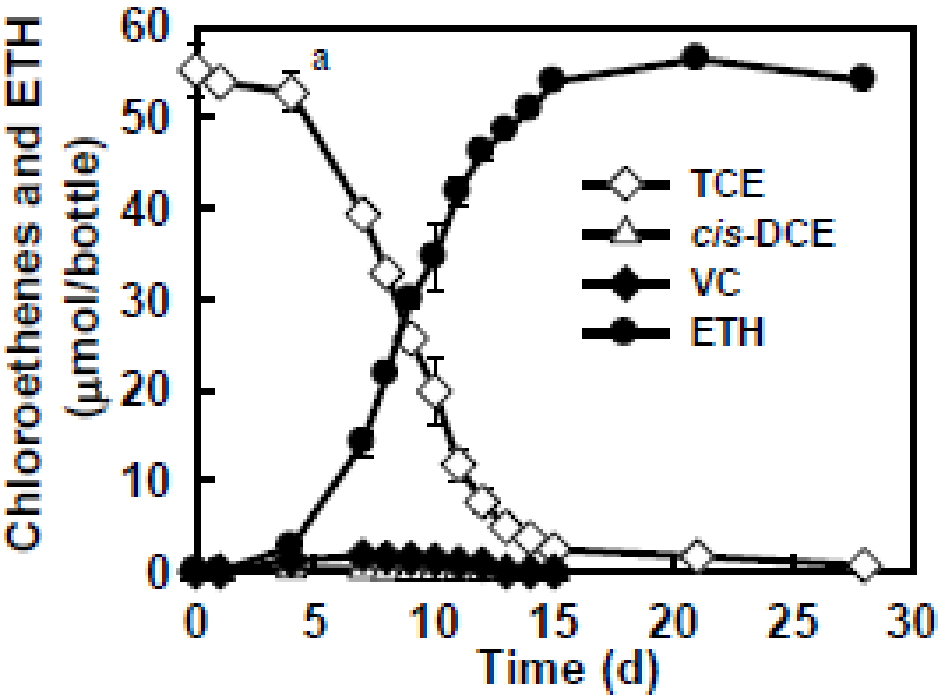
Several companies worldwide now use *Dehalococcoides*-containing mixed cultures in commercial remediation efforts. In mixed cultures, other bacteria present can augment the dehalogenation process by producing metabolic products that can be used by *Dehalococcoides*and others involved in the degradation process.[9][16] For example, *Dehalococcoides* sp. strain WL can work alongside *Dehalobacter* in a step-wise manner to degrade vinyl chloride: *Dehalobacter* converts 1,1,2-TCA to vinyl chloride, which is subsequently degraded by *Dehalococcoides*.[17] Also, the addition of electron acceptors is needed - they are converted to hydrogen *in situ* by other bacteria present, which can then be used as an electron source by *Dehalococcoides.*[12][9] MEAL (a methanol, ethanol, acetate, and lactate mixture) is documented to have been used as substrate.[18] In the US, BAV1 was patented for the *in situ* reductive dechlorination of vinyl chlorides and dichloroethenes in 2007.[19] *D. mccartyi* in high-density dechlorinating bioflocs have also been used in *ex situ* bioremediation.[20]

Although *dehalococcoides* have been shown to reduce contaminants such as PCE and TCE, it appears that individual species have various dechlorinating capabilities which contributes to the degree that these compounds are reduced. This could have implications on the effects of bioremediation tactics.[13] For example, particular strains of *Dehalococcoides* have shown preference to produce more soluble, carcinogenic intermediates such as 1,2–dichloroethene isomers and vinyl chloride that contrasts against bioremediation goals, primarily due to their harmful nature.[4][8] *Dehalococcoides mccartyi sp.* MB dechlorinated PCE and TCE to *trans*-DCE predominantly and *cis*-DCE as a minor end product.

Dehalococcoides mccartyi sp. strain 11a, however, was discovered to be able to dechlorinate TCE, trans- and cis-DCE, 1,1-DCE, and VC metabolically to ethene without any accumulation of intermediates, through the *vcrA* gene. Strain 11a shares 100% 16S rRNA gene sequence identity with the first VC-dechlorinating isolate Dehalococcoides sp. strain BAV1, but differs in the reductive dehalogenases used (VcrA for 11a and BvcA for BAV1). The *vcrA* gene of culture 11a also shows a difference of nine and ten base pairs (bp) from strain ANAS2 and VS, respectively. This may account for 11a's rapid dechlorination rate and its diverse substrates for dechlorination compared to other *vcrA*-containing cultures. This strain 11a could also dechrinated 1,2-dichloromethane to ethene without any accumulation of toxic intermediates. Complete detoxification of PCE to ethene in contaminated groundwater was achieved within 10 days by the co-culture of strain 11a and other PCE-dechlorinating bacteria, *Sulfurospirillum multivorans*. It provides promising application of the new isolate (11a) in bioremediation of chloroethene-contaminated sites.

Among the potential electron acceptors tested**,** *Dehalococcoides* sp. **s**train 11a dechlorinated TCE, 1,1-DCE, *trans*-DCE, *cis*-DCE, VC, and 1,2-DCA to ethene with an average rate of 53.1, 22.5, 21.6, 24.8, 86.5, 16.7 µmol L^{-1} day^{-1}, respectively (Fig 1). It shows that culture 11a could dechlorinate TCE (Fig 1a) in about two weeks with little accumulation of VC (less than 2 µmols), whereas there was no accumulation of VC for *trans*-DCE dechlorination (Fig 1b). The fast TCE-dechlorination process indicates its promising application for bioremediation.

Like other *Dehalococcoides* species, strain 11a uses acetate as the carbon source, and H_2 as the electron donor and cannot utilize lactate, pyruvate, propionate, glucose, succinate, or glutamate for growth.


a
TCE
cis-DCE
VC
ETH
Chloroethenes and ETH (μmol/bottle)
Time (d)
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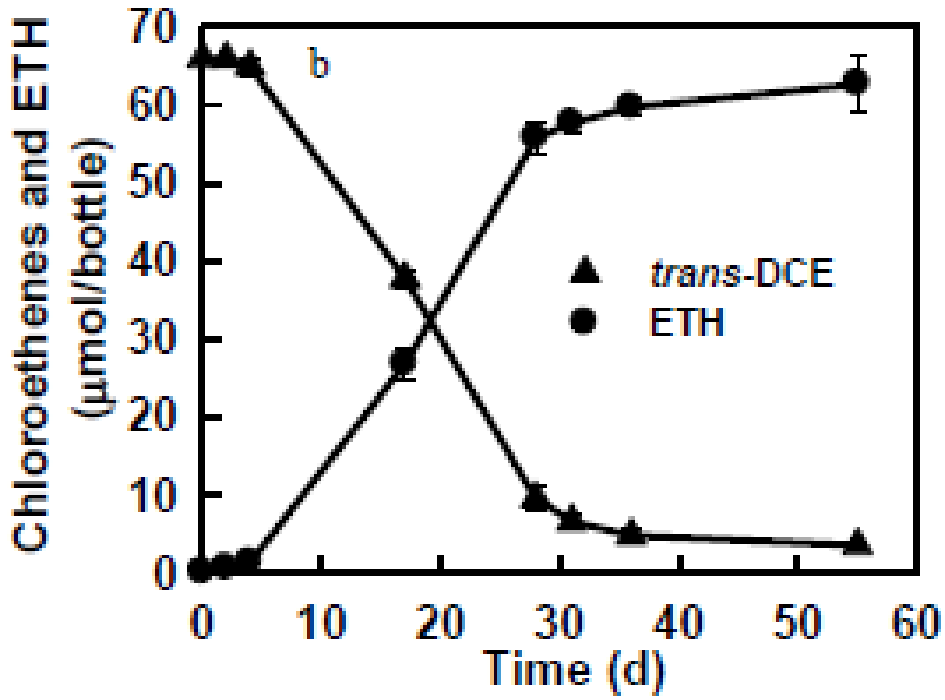
b
trans-DCE
ETH
Chloroethenes and ETH (μmol/bottle)
Time (d)
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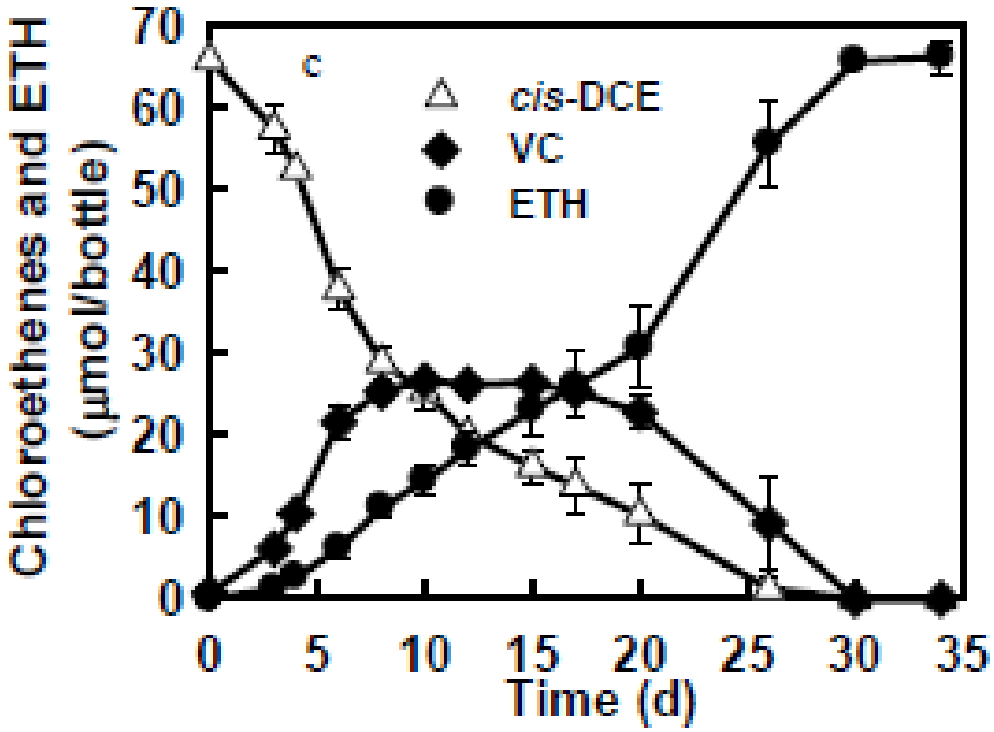
c
cis-DCE
VC
ETH
Chloroethenes and ETH (µmol/bottle)
Time (d)
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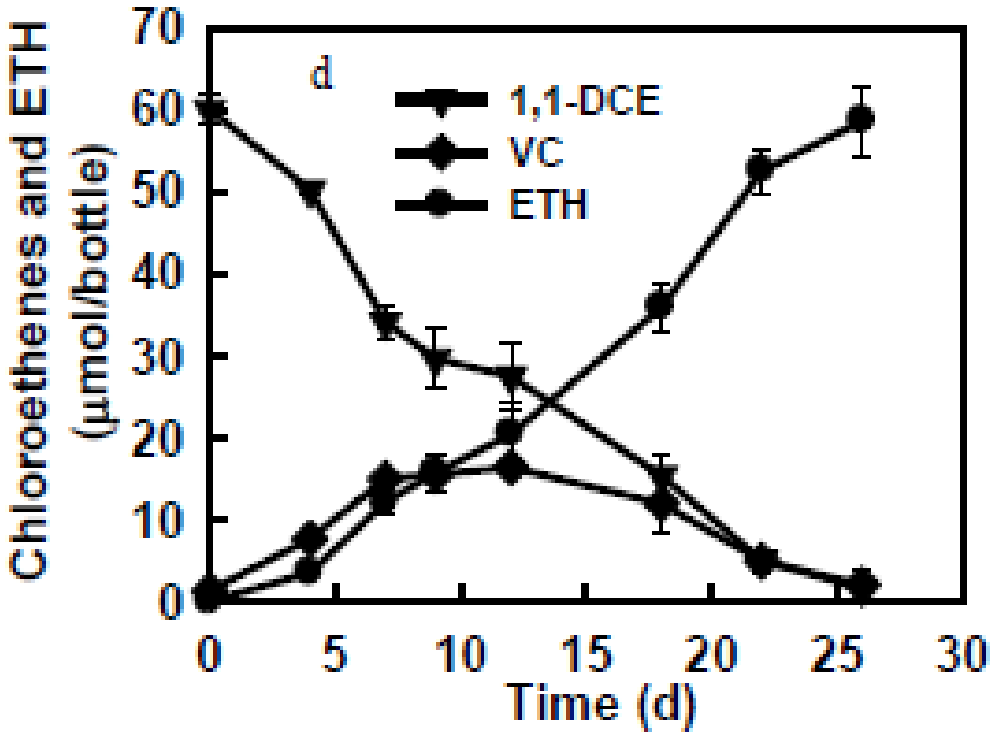
d
1,1-DCE
VC
ETH
Chloroethenes and ETH (µmol/bottle)
Time (d)
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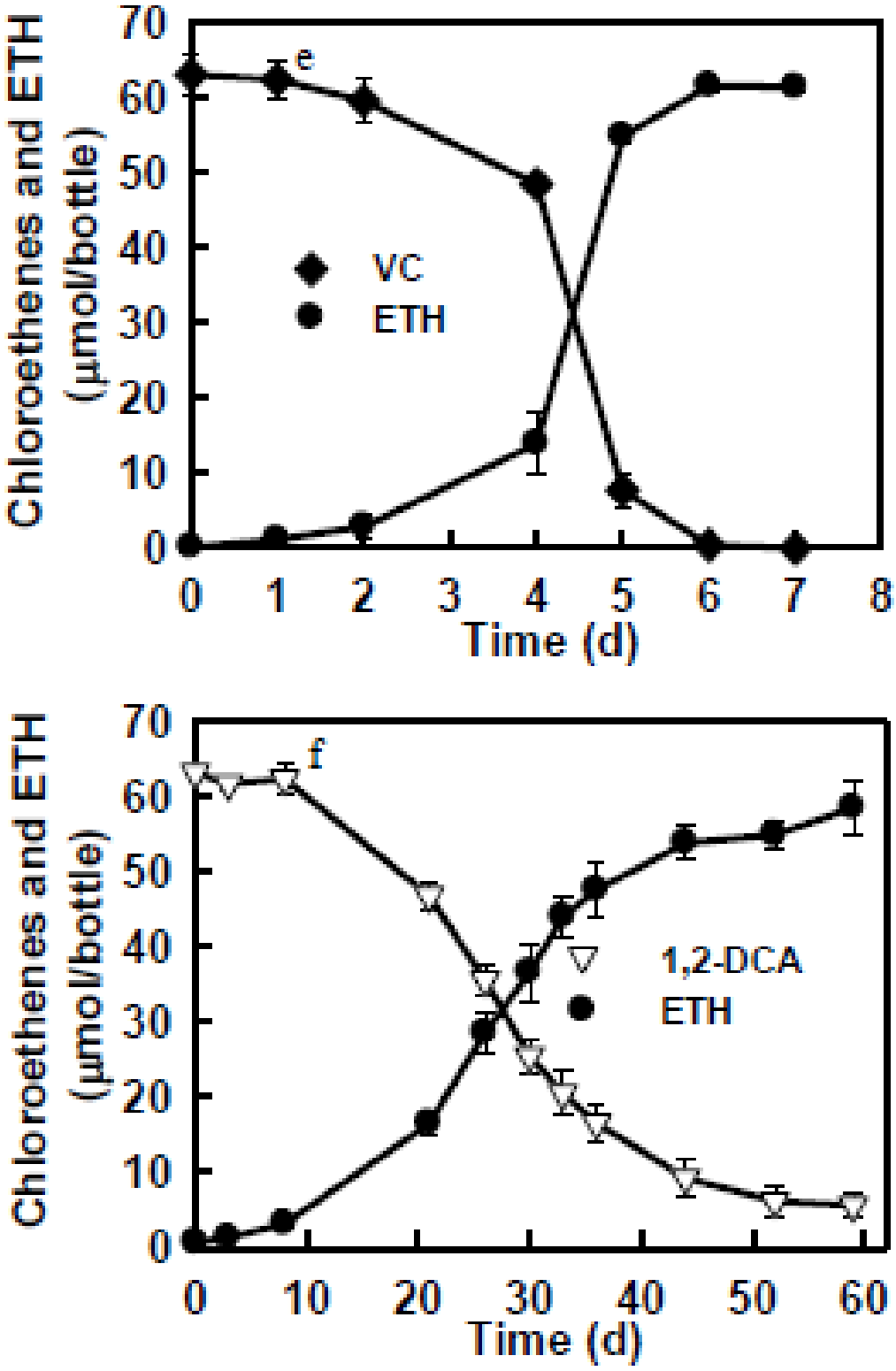
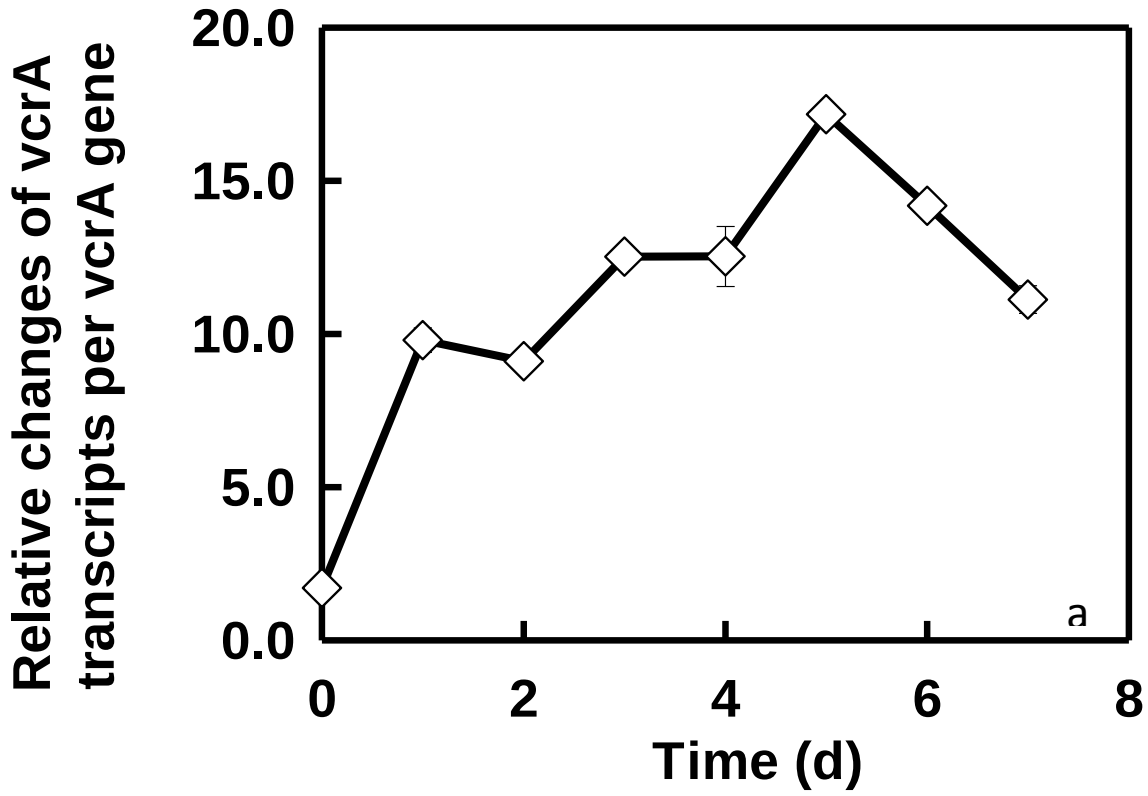


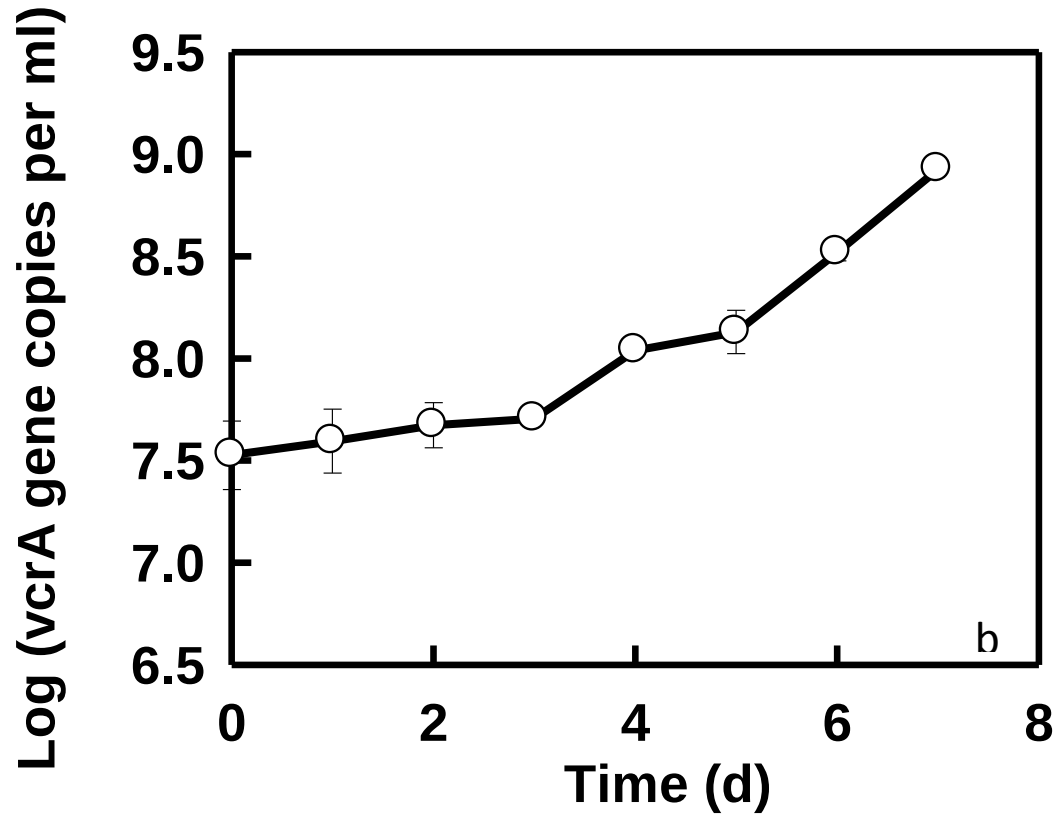
Fig. 1 Reductive dechlorination of halogenated compounds by *Dehalococcoides* sp. strain 11a. (a) TCE, (b) *trans*-DCE, (c) *cis*-DCE, (d) 1,1-DCE, (e) VC, (f) 1,2-DCA. Data points were averaged from triplicate cultures, and error bars represent standard deviations. Like other *Dehalococcoides* species, strain 11a uses acetate as the carbon source, and H_2 as the electron donor and cannot utilize lactate, pyruvate, propionate, glucose, succinate, or glutamate for growth.

Role of VcrA in strain 11a during dechlorination of TCE, *trans*-DCE and VC

During the complete dechlorination of VC (Fig. 2c), maximal expression of *vcrA* gene was found at day 5 (Fig. 2a) which is close to the end of the exponential phase (Fig. 2b). After day 5, a slight decrease was observed for the transcription level of *vcrA*, probably due to the faster increase in the DNA copies (intensive growth of culture VT with VC) when dechlorination of VC was nearly completed.

Before dechlorination activity was observed, the transcriptional analysis using substrate TCE and VC shows that the *vcrA* mRNA in pure culture 11a were highly expressed within 24 h and reached the expression maximum after 12 h's exposure to TCE and VC. It is known that *vcrA* gene is identified to be responsible for VC dechlorination (Müller et al., 2004). The transcript levels of *vcrA* gene exposed to TCE were found to be 4-fold higher than that of VC (Fig. 2c), suggesting that the *vcrA* gene is functionally important for the respiration of both TCE and VC.





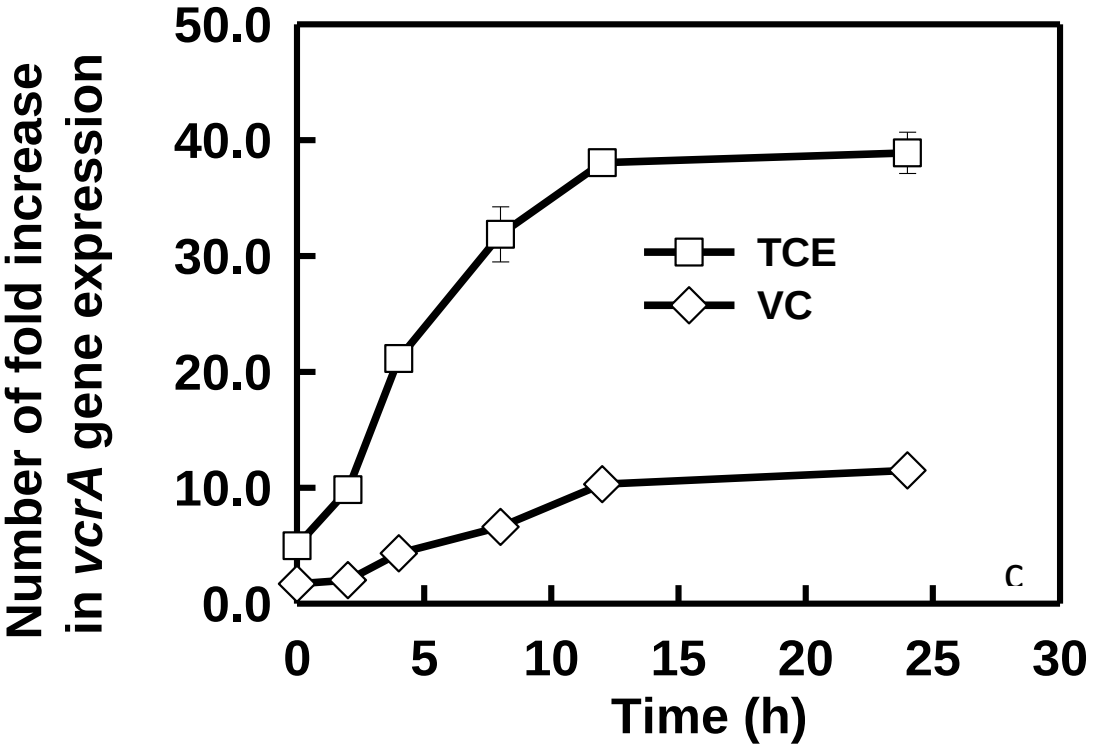


Fig. 2 The role of vcrA gene during reductive dechlorination of TCE and VC by *Dehalococcoides* sp. strain 11a. (a) Gene expression of *vcrA* gene with TCE and VC, (b) change of transcript numbers of

vcrA for strain 11a, (c) growth of culture 11a during 7-day dechlorination of VC. Data points were averaged from triplicate runs, and error bars represent standard deviations.

The role of VcrA in dechlorination of *cis*-DCE or 1,1-DCE was proven previously (Müller et al., 2004), whereas the discrepancy occurs for *trans*-DCE that active enzyme VcrA could catalyze the *trans*-DCE dechlorination whereas the active *vcrA*-containing culture (e.g. VS) never shows this dechlorination capability. The purified enzyme VcrA showed dechlorination ability for *trans*-DCE, however the previously found *vcrA*-containing active cultures failed to use this substrate including culture VS, KB-1, GT or ANAS. The SDS-PAGE was conducted to check the role of VcrA in the active *trans*-DCE dechlorinating culture 11a as compared to TCE. Fig .3 shows that the same major band (~ 62 kDa) was found to be have highest expression during the reductive dechlorination of TCE and *trans*-DCE. The position of this expressed 62-kDa band agrees well with the VC-dechlorinating enzyme, VcrA found in culture VS (Müller et al., 2004), suggesting the functional role of VcrA of strain 11a in dechlorination of not only VC, but also TCE and *trans*-DCE. This versatile dechlorinating activity was consistent with the activity of in vitro assay of VcrA purified from mixed culture VS (Müller et al., 2004) and agreed well with the cell growth measured by qPCR during the dechlorination of *trans*-DCE by 11a. The results of this study showed that the highly expressed RDase (VcrA) in culture 11a has broader substrate range than that of culture VS or GT in that neither of these active cultures showed metabolic activity for *trans*-DCE. In other words, the SDS-PAGE results together with 11a's physiology and qPCR results confirm that the VcrA in culture 11a encodes the dechlorination of VC, all DCEs isomers and TCE to ethene.

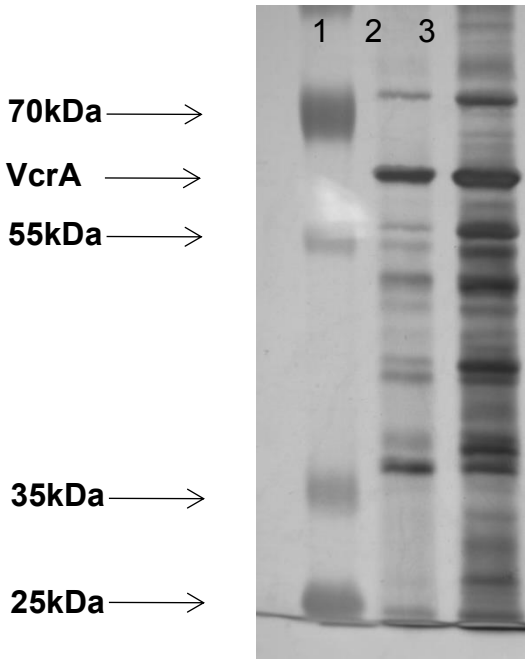


Fig. 3 SDS-polyacrylamide gel of VC-reductive dehalogenase of *Dehalococcoides* sp. strain 11a. Lane 1, prestained pageruler protein ladder; lane 2 and 3, active cell extract of *trans*-DCE and TCE, respectively (after gel filtration with sizes of major peptides).

To warrant successful bioaugmentation, it would be necessary to implement the culture like 11a, capable of dechlorinating not only TCE, but also *trans*-DCE, which tend to accumulate at contaminated sites and not well detoxified by other strain of *Dehalococcoides*.

A new set of Taqman primers and probe was designed to quantify *vcrA* gene: forward primer vcrA11F (5'-GTA TGG TCC GCC ACA TGA TTC-3'), reverse primer vcrA11R (5'-TCT TCT GGA GTA CCC TCC CAT TT-3'), and probe vcrA11P (5'-FAM-CGC CAC CTG ATG GGA GCG TAC C-TAMRA-3'). This new set of Taqman-based primers and probe was designed to target various kinds of vcrA gene in order to cover a number of other VC-dechlorinating microbes, e.g. KB-1 like culture, ANAS enrichment culture, VS and GT.

Therefore, an important aspect of current bioremediation tactics involves the utilization of multiple dechlorinating organisms to promote symbiotic relationships within a mixed culture to ensure complete reduction to less-toxic ethene.[13] As a result, studies have focused upon metabolic pathways and environmental factors that regulate reductive dehalogenative processes in order to better implement *Dehalococcoides* for bioremediation tactics.[8]

Fig 4 and Fig. 5shows that under optimized conditions, co-culture of strain 11a and strain *S. multivorans* were able to completely detoxify PCE to ethene in the collected contaminated ground water within 10 days

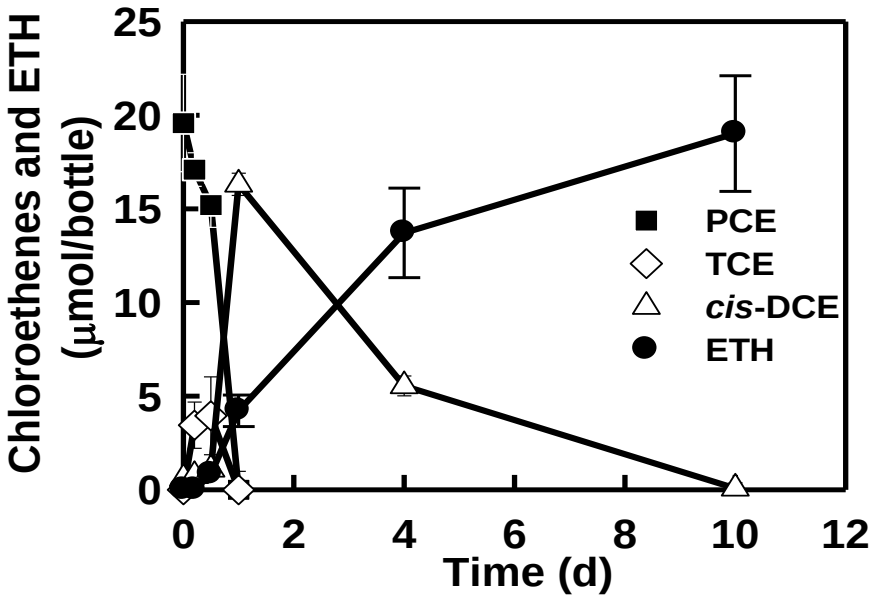


Fig. 4. Bioremediation of PCE-contaminanted groundwater by *Sulfurospirillum multivorans* and *Dehalococcoides* sp. 11a.

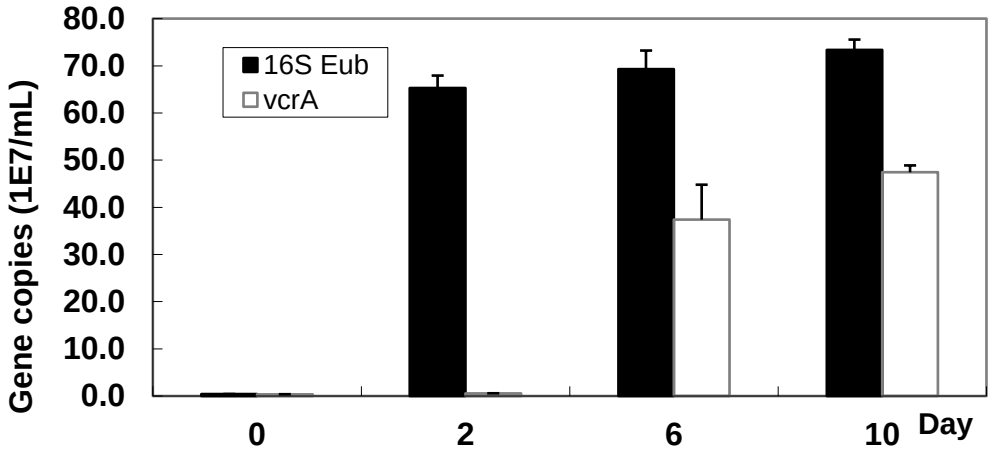


Fig. 5 Relative abundance of *Dehalococcoides* mccartyi sp. 11a in the co-culture (with *Sulfurospirillum multivorans*) during the remediation of contaminated groundwater as revealed by qPCR.

Bear in mind, not all members of *Dehalococcoides* can reduce all halogenated contaminants. Certain strains cannot use PCE or TCE as electron acceptors (e.g. CBDB1) and some cannot use vinyl chloride as an electron acceptor (e.g. FL2).[14] *D. mccartyi* strains 195 and SFB93 are inhibited by high concentrations of acetylene (which builds up in contaminated groundwater sites as a result of TCE degradation) via changes in gene expression that likely disrupt normal electron transport chain function.[9] When selecting *Dehalococcoides* strains for bioremediation use, it is important to consider their metabolic capabilities and their sensitivities to different chemicals.

Protocols for setting up microcosoms in lab

This protocol consists of two steps, sampling at site and subsequent setting up of microcosms.

A. Sampling at sites.

Choose those sites which may be contaminated by TCE or PCE before. Collect some sludge sample into a sealed container and bring it back to the anaerobic chamber ASAP.

1) Set the targeted sites first, and prepare enough 50-ml sterile falcon tubes (at least 2-3 tubes per site) and label them with permanent marker. 2) Bring spatula, 70% ethanol spray bottle, gloves, tissue, water (for wash hands after sampling-optional), and bags for solid samples to the sites. For liquid samples, it may be helpful to bring the pipette (10-ml) or autoclaved sealed duran bottles flushed with N2 as headspace. Label it properly. Bring an ice box for temporary storage if possible. 3) During sampling, ensure minimal exposure to oxygen, e.g., For liquid samples, fill tubes up as full as possible with liquid samples and if possible, avoid capping tubes in air. For solid samples, fill up tube with liquid sample or ultrapure water (for solid, dry sample). Minimize cross contamination between samples as much as possible. 4) Keep the microcosms at 4 °C when back to the lab. Setting up the microcosms as soon as possible. If not, better to freeze the samples at -20 °C.

Before experiment, check whether the anaerobic chamber is in good condition for working and the outlet pressure for both N_2 and anaerobic mixed gas should be at 10 psi.

B. Setting up microcosms at lab

1) Wash enough serum bottles (according to the number of microcosms) with ultrapure water and dry it in the oven at about 100 °C for 1 h. Rinse the black stoppers, put it into a glass beaker

then dry it at 60-70 °C for 2 h. 2) Cover the serum bottles (or 60 ml serum bottles) individually and the beaker (containing stoppers) with aluminum foil. Then go to autoclave. Be reminded to set the control bottles together with the sampling bottles in duplicate). After autoclave, mark the serum bottles and label them on the tape properly. 3) Prepare 2000 ml of medium according to Table 4 (at least 120 ml of medium for each microcosm). Under flushing of N2 (minimum flow rate, about 1 psi), bring the medium solution to boil fully (100 °C). Once the solution starts to boil, let it boil for another 10min before removing it from the heater.

4) Cool the medium solution to room temperature under higher flow rate of N2 (about 4-5 psi). Next, add the reducing agents in Table 5 to the medium solution quickly so as not to introduce too much O_2 into the medium.

5) Mix the solution (using magnetic stir bar) to fully dissolve the chemicals. Medium should turn colourless.

6) Insert a pH probe into the solution. Adjust the pH through different flowrate of N2/CO2. Let the pH rise until 7.2 – 7.3.

7) When pH meter shows the desired pH, seal the three-neck round bottom flask with stoppers (rubber or glass) and parafilm the necks to maintain anaerobic conditions. Next, autoclave the flask at 121°C, 20 min, 210 kPa.

8) Once done, transfer the flask along with the necessary items: e.g. spatula, tissue, oxygen indicator, 60-ml syringe for media dispensing, catalyst panel and a small beaker, 70% ethanol spray bottle, gloves (one pair of gloves for each microcosm to prevent cross-contamination), autoclaved serum bottles, aluminum caps, black butyl rubber stoppers, crimper, biohazard/trash bag, vitamin inside the disposable syringe, paper for the working bench, and other stuff deemed necessary) into the anaerobic chamber. Loosen the caps of spray bottle (ethanol) or water bottle if applicable. Punch holes on aluminum foils for serum bottles/stopper beaker with disposable needle to allow residual O2 in the bottles to be vacuumed.

9) Check whether the water level inside the anaerobic chamber in between the 2 black lines. If not, adjust the water level by removing extra water or adding necessary amount of ultra pure water.

10) Place them into the side chamber and close the door, then press “autocycle”. Once ready, the chamber panel will indicate “anaerobic” (light on). Then place two arms inside the gloves and ensure good sealing (no leakage). Use foot to replace the air inside the arm with N2 or mixed gas by stepping on the “Vacuum” followed by “Gas” foot panel. Repeat this cycle for 2-3 times. Then enter the main chamber with two arms by gently loosing the two front doors simultaneously.

11) Once enter the main anaerobic chamber, replace the present “old” catalyst in the anaerobic chamber with the “new” one. Remove the extra water from the small beaker inside the main chamber or change a new beaker.

12) Open the oxygen indicator. Wait until it turns relatively clear then start work inside the chamber. During this process, put one layer of bench-work paper inside the chamber to maintain the cleanliness of the main chamber. Wipe the working area with 70% alcohol.

13) Sterilize gloves. Add the required amount of filter-sterilized vitamins directly into the medium in the three-neck flask and gently shake to ensure minimal agitation (to reduce the chance for media to turn pink). Transfer (about 10 g if solid and 10 ml if liquid) microcosm material (e.g. soil, sediment) into the autoclaved serum bottles using spatula (solid) or syringe/manually pouring (liquid). Sterilize the gloves or change to a new pair of gloves when handling with different microcosms.

14) Add the required amount of medium (about 25-ml for 60-ml serum bottle, 40-100 ml for 160 ml serum bottle) into the serum bottles using a 60-ml syringe and seal with rubber septa and finally crimp using crimper. It is advisable to allow the medium in the flask to ideally turn clear before dispensing to the serum bottles INSIDE the anaerobic chamber.

15) When done, remove everything that does not belong in the chamber (e.g. any trash and old catalyst panel) and clean up any mess.

16) Add the substrate in the fume hood (outside the chamber) if the substrate is toxic, e.g. TCE (with disposable syringe and needles).

How to prepare medium in lab to grow *Dehalococcoides* spp. anaerobically

I. Reagents

Table 1: Trace element solution (1000×)

Chemicals used	Amount	
	ml	g
HCl (25% solution, w/w)	10	-
$FeCl_2 \cdot 4H_2O$	-	1.5
$CoCl_2 \cdot 6H_2O$	-	0.19
$MnCl_2.4H_2O$	-	0.1
$ZnCl_2$	-	0.07
H_3BO_3	-	0.006
$Na_2MoO_4 \cdot 2H_2O$	-	0.036
$NiCl_2 \cdot 6H_2O$	-	0.024
$CuCl_2 \cdot 2H_2O$	-	0.002

Table 2: Se/W Solution (1000×)

Chemicals used	Amount	
	ml	g
$Na_2SeO_3 \cdot 5H_2O$	-	0.006
$Na_2WO_4 \cdot 2H_2O$	-	0.008
NaOH	-	0.5

Table 3: Salts Solution (100×)

Chemicals used	Amount (1 x g/L)	Amount (100xg/L)	Amount (g/100ml)
NaCl	1.0	100.0	10.0
$MgCl_2 \cdot 6H_2O$	0.5	50.0	5.0
KH_2PO_4	0.2	20.0	2.0
NH_4Cl	0.3	30.0	3.0
KCl	0.3	30.0	3.0
$CaCl_2 \cdot 2H_2O$	0.015	1.5	0.15

Table 4: Medium Solution (acetate 5 mM) for 1000 ml

Chemicals used	Amount (1L)	
	ml	g
100 x salt solutions	10	-
Trace element **(1000×)**	1	-
Se/W Solution **(1000×)**	1	-
TES (10mM)	-	2.292
Resazurin (0.1% solution)	0.25	-
Sodium acetate (5 mM)	-	0.6804
milli-Q water	Top up	-

Table 5: Reductants and buffering agent

Chemicals used	Amount (1L)	
	ml	g
0.2mM L-cysteine	-	0.0242
0.2mM $Na_2S \cdot 9H_2O$	-	0.048
0.5mM DL-dithiothreitol (DTT)	-	0.0771
30mM $NaHCO_3$	-	2.52

Table 6: Vitamin and Vitamin B_{12} Solution (1000×)

Chemicals used	Amount (1L)	
	ml	mg
Biotin	-	20
Folic acid	-	20
Pyridoxine Hydrochloride	-	100
Riboflavin	-	50
Thiamin	-	50
Nicotinic acid	-	50
Pantothenic acid	-	50
p-aminobenzoic acid	-	50
Thioctic acid	-	50
Vitamin B_{12}	-	1

B_{12}: 1000× (50mg/ml) 1× (50µg/ml)

Table 7: Preparation of additional vitamin B12 solution

Vitamins	1000x mg/L	Final Conc. (mg/L)	200x mg/L
Vitamin B12	50mg	0.05	0.01mg

How to prepare the medium

Step 1: Preparation of medium solution

Prepare trace element solution according to Table 1. Prepare Se/W solution according to Table 2. Prepare 100ml salt solution according to Table 3. Prepare 200ml of medium according to Table 4. Under flushing of N2 (minimum flow rate), bring the medium solution to boil fully (100 degree C). Once the solution starts to boil, let it boil for another 10min before removing it from the heater. Cool the medium solution to room temperature under higher flow rate of N2. Next, add the chemicals in Table 5 to the medium solution quickly so as not to introduce too much O2 into the medium. Shake the solution to fully dissolve the chemicals. Medium should turn colourless. Insert a pH probe into the solution. Let the pH rise until 7.2 – 7.3. Adjust the pH through different flowrate of N2/CO2. Flush the test tubes with N2/CO2 (80/20). Flush the syringe

used for dispensing by drawing N2/CO2 from the medium solution 2 to 3 times. Once the pH rises to 7.2, start to dispense 9ml of medium into test tubes. Take note not to draw air into the syringe. Close the tubes with blue rubber stopper and crimped sealed with aluminium caps to ensure no leakage. Maintain the pH of the medium during dispensing to be 7.2-7.3 by flushing with N2/CO2 (90/10) once the pH rise above 7.3. Autoclave the test tubes at 121oC, 20min, 210 kPa. The medium solution should be clear after autoclave. Discard any medium solution that is pink a day after autoclave.

Step 2: Preparation of vitamins solution and vitamin B12

Prepare a 200x vitamins solution according to Table 6. Also, prepare a 200x vitamin B12 solution by dissolving 0.02mg in 1L of DI water Shake well to dissolve the contents. Use aluminium foil to wrap the bottle as the vitamins solution are light sensitive. Adjust the pH of the vitamins solution using 10M NaOH to 7.5 Prepare two 160ml bottles flushed with N2, close with rubber stopper and sealed with aluminium cap, autoclave at 121oC, 20 min, 210kPa Due to heat sensitivity of the vitamins solution, sterilise the vitamins solution and vitamin B12 by filtering the solution through a sterile filter into the autoclaved 160ml bottle (flushed with N2). Wrap the two 160ml bottle filled with sterilized vitamins solution and vitamin B12 with aluminium foil and store in refrigerator.

Step 3: Addition of vitamins solution and vitamin B12 and TCE to the medium test tubes

Perform the addition of vitamins solution in the class II biosafety cabinet. "On" the biosafety cabinet and ensure that the air flow is stable (green region) Swap the working surface with 70% ethanol to disinfect. Prepare a bottle flushed with N2, autoclaved for reducing. Disinfect the surface of the 2 bottle of vitamins solution and vitamin B12 with 70% ethanol, flame the ethanol swapped surface to burn off completely. Repeat the disinfection step in step 5 for the medium test tubes. Using disposable 1ml syringe attached to a new needle (let it be needle 1), reduce the syringe 2-3 times by drawing N2 from the autoclaved bottle. Invert the vitamins solution and insert the needle fully through the rubber stoppers and draw 1ml of vitamin solutions. Remove the needle and attach the same syringe to a sterile 0.2µm filter with a new needle (let it be needle 2). Filter the vitamins solution in the syringe through the sterile filter. Detach the sterile filter and needle (taking care not to contaminate) and fix the same syringe back to needle 1. Invert the vitamins solution bottle and draw another 1ml. Detach needle 1 and fix to previous sterile filter attached to needle 2. Push the syringe to get rid of any air bubbles trapped. Invert the vitamins solution bottle and insert needle 2 into the medium test tubes and add 0.05ml to each test tubes. Invert the test tubes immediately after withdrawing the needle to minimize possible leakage of the rubber stopper. After the addition of vitamins, proceed to add TCE to each of the medium test tubes. Perform the addition of TCE in the fume hood (ensure exhaust is working well). Add a drop (equivalent to 2µl TCE) into each of the medium test tubes. Label the test tubes according to the no. of drops of TCE added. Leave the test tubes overnight at room temperature and observe for any changes in medium colour to pink.

Step 4: Inoculation of mixed culture to the medium test tubes

Proceed the inoculation for those test tubes that remain clear after overnight (indication of absence of O2) Prepare 2 sets of medium test tubes for duplicate results until dilution (-6). Total no. of medium test tubes needed is 12. Perform the inoculation in the biosafety cabinet "On" the biosafety cabinet and ensure that the air flow is stable (green region) Swap the working surface with 70% ethanol to disinfect. Using disposable 1ml syringe attached to a new needle, reduce the syringe 2-3 times by drawing N2 from the autoclaved bottle. Disinfect the surface of the mixed culture bottle with 70% ethanol, flame the ethanol swapped surface to burn off completely. Repeat the disinfection step in step 6 for the medium test tubes. Invert the mixed culture bottle and draw 2ml of mixed culture with the syringe. Inoculate 1ml each into two medium test tubes. Invert the inoculated test tubes a few times to mix well. Using a new syringe and needle, invert the first inoculated test tube and withdraw 1ml of solution and transfer to the 2nd medium test tube (dilution 10-1). Invert the 2nd medium test tube a few times before drawing 1ml for transfer to the 3rd test tube. Continue the serial dilution step for the rest of the medium test tubes using

the same syringe and needle until dilution 10-6. Label the dilution factor on the test tube. Repeat step 11 to 13 for the duplicate set. Incubate the inoculated test tubes at 30oC with the stopper down.

Step 5: Using Gas Chromatograph (GC) equipped with FID to detect the activity of anaerobic bacterium

Chorinated aliphatic hydrocarbons including PCE, TCE, cis-TCE, trans-DCE, 1,1-DCE were purchased from Sigma-Aldrich-Fluka. Prepare a standard MIX containing all the 5 chemicals above by adding 2µl of each chemical into the same autoclaved blank medium. Prepare another 5 standards by adding 2µl of each chemical individually into each bottle. By trial and error, develop a method in the GC which can detect the chemicals present in the standards. Using a sterile 1ml syringe, draw suitable volume (e.g. 50µl or 100µl depending on the concentration) from the headspace of the standard MIX into the GC and record down the retention time, peak area and peak height. Repeat the same for the other individual standards of the 5 chemicals. Measure the initial concentration of the TCE in the headspace of the medium bottles by drawing suitable volumes for GC analysis. Monitor the dechlorinating activity of the inoculated medium bottles weekly by GC-FID analysis. Compare the results with the standards.

Note: this protocol can be used for any Dehalococcoides spp.

How to obtain *Dehalococcoides* isolates

Series dilution to extinction method in both liquid medium and agar shake. The key to obtain the pure culture is to apply antibiotic (ampicillin, 100 µg/mL) to the medium before inoculation, both liquid and solid agar. Note: generally this approach could result in the isolation within 6 months, like strain 195, CBDB1, BAV1, FL2, 11a*, 11a5, ANAS1, ANAS2, SG1 etc.

Table 4. The functional genes of various *D. spp*. strains for its dechlorinated compounds and end products （Ref 25）.

Strain	Functional genes	Dechlorinated compounds	End products	References
*D. mccartyi*195	*pceA, tceA*	PCE, TCE, *cis*-DCE, 1,1-DCE	Ethene	[30], [56], [73], [74]
		1,2-dichloroethane	VC, Ethene	
		1,2,3,4-tetrachlorodibenzo-p-dioxin	1,2,4-trichlorodibenzo-p-dioxin, 1,3-dichlorodibenzo-p-dioxin	
		Hexachlorobenzene	1,2,3,5-tetrachlorobenzene, 1,3,5-trichlorobenzene	
		2,3,4,5,6-chlorobiphenyls (CB)	2,3,4,6-CB, 2,3,5,6-CB, 2,4,6-CB	

Strain	Functional genes	Dechlorinated compounds	End products	References
		2,3-DCP, 2,3,4-TCP, 2.3,6-TCP	Lower chlorinated phenols	
*D. mccartyi*CBDB1	*cbrA*	1,2,3-trichlorobenzene (TCB), 1,2,4-TCB, 1,2,3,4-TeCB, 1,2,3,5-TeCB and 1,2,4,5-TeCB	1,3-DCB, 1,4-DCB, and 1,3,5-TCB	[75], [76], [77]
*D. mccartyi*VS	*vcrA*	*cis*-DCE & VC	Ethene	[43], [44], [46]
*D. mccartyi*BAV1	*bvcA*	*cis*-DCE, trans-DCE, 1,1-DCE, VC, Vinyl bromide, 1,2-dichloroethane	Ethene	[45], [46]
*D. mccartyi*FL2	*tceA*	TCE, *cis*-DCE & *trans*-DCE	VC & Ethene	[45], [78]
*D. mccartyi*KB1/VC	*tceA*	TCE, *cis*-1,2 DCE & VC	Ethene	[59], [63]
*D. mccartyi*GT	*vcrA*	TCE, *cis*-DCE, 1,1-DCE, VC	Ethene	[64]
*D. mccartyi*DCMB5	*cbrA*	1,2,3,4-tetrachlorodibenzo-p-dioxin	2-monochlorodizenbo-p-dioxin	[79], [80]
*D. mccartyi*MB	*mbrA*	PCE & TCE	*trans*-DCE, *cis*-DCE	[33], [34]
*D. mccartyi*BTF08	*pceA*, *tceA*, *vcrA*	PCE, TCE, *cis*-DCE, & VC	Ethene	[41], [80]
*D. mccartyi*ANAS1	*tceA*	TCE, 1,1-DCE, & *cis*-DCE	VC & Ethene	[33]
*D. mccartyi*ANAS2	*vcrA*	TCE, *cis*-DCE, 1,1-DCE, & VC	Ethene	[56], [66]
*D. mccartyi*11a	*vcrA*	TCE, *trans*-DCE, *cis*-DCE, 1,1-DCE, 1,2-DCA, & VC	Ethene	[56]
*D. mccartyi*11a5	*tceA*	TCE, *trans*-DCE, *cis*-DCE, & 1,1-DCE	VC & Ethene	[56]
*D. mccartyi*IBARAKI	*vcrA*	*cis*-DCE & VC	Ethene	[47]
*D. mccartyi*UCH007	*pceA*, *tceA*, *vcrA*	TCE, *cis*-1,2-DCE & VC	Ethene	[42]

Strain	Functional genes	Dechlorinated compounds	End products	References
*D. mccartyi*CG1	*pcbA1*	PCE, 234-234-CB, 234-24-chlorinated biphenyls (CB)	TCE, 24-24-CB, 24-25-CB, 235-24-CB,236-24-CB	[35]
*D. mccartyi*CG4	*pcbA4*	PCE, 2345-, 2346-, and 245-CB, 23456-, 2345-, 245-, and 234-CB	TCE, 24-24-CB, 24-25-CB	[35]
*D. mccartyi*CG5	*pcbA5*	PCE, 2345-, 234-, 235-, 236-, and 245-CB, 2345-, 2346-, and 245-CB	TCE, 24-24-CB, 24-25-CB, 25-26-CB, 235-24-CB, 236-24-CB, 245-24-CB	[35]
*D. mccartyi*JNA	*pcbA4*, *pcbA5*, *pceA*, *mbrA*	Pentachlorophenol 2,2,4,6-tetrachlorophenol, 2,4,5-trichlorophenol (TCP) 2,3-DCP	3,5-dichlorophenol (DCP) 2,4,6-(TCP) 2,4-DCP, 3,4-DCP, 3-chlorophenol (CP)	[81]
*D. mccartyi*GY50	NA[a]	NA[a]	NA[a]	[42], [82]
*D. mccartyi*SG1	NA[a]	NA[a]	NA[a]	[35], [42]

***Note: *Dehalococcoides mccartyi* strain 11a and strain GEO are interchangeable. These two strains have similar function in dechlorinating TCE.**

Genomes[edit]

Several strains of *Dehalococcoides sp.* has been sequenced.[21][22][23] They contain between 14 and 36 reductive dehalogenase homologous (rdh) operons each consisting of a gene for the active dehalogenases (rdhA) and a gene for a putative membrane anchor (rdhB). Most rdh-operons in *Dehalococcoides* genomes are preceded by a regulator gene, either of the marR-type (rdhR) or a two-component system (rdhST). *Dehalococcoides* have very small genomes of about 1.4-1.5 Mio base pairs. This is one of the smallest value for free-living organisms.

Biochemistry[edit]

Dehalococcoides strains do not seem to encode quinones but respire with a novel protein-bound electron transport chain.[24]

See also[edit]

- Bioaugmentation

- Bioremediation
- Biostimulation

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