Article ID: 0253-2778(2009)01-0083-07

Physiological characterization of a microbial consortium that reductively dechlorinates 1,1-dichloroethane to chloroethane

YANG Rui, SUN Bao-lin

(*Hefei National Laboratory for Physical Sciences at Microscale, and School of Life Sciences, USTC, Hefei* 230027, *China*)

Abstract: An anaerobic microbial consortium reductively dechlorinating 1,1-dichloroethane (DCA) to chloroethane (CA) was enriched from the sediment of Hudson River, New York. The consortium used hydrogen, formate, acetate, benzoate and fumarate as electron donors for reductive dechlorination. Pyruvate fermentation occurred in the enrichment culture, although this compound was not used as an electron donor for dechlorination. Among other halogenated compounds tested, only DCA was reductively dechlorinated. Sulfite could completely inhibit DCA dechlorination, whereas sulfate, nitrate or fumarate had no effect. Growth by reductive dechlorination with hydrogen as electron donor was revealed by a cell yield of (4.402±1.241)g of cells (dry weight) per mole of chloride released. Sequence analysis of 16S rRNA gene amplified from a highly enriched culture with H_2 as electron donor suggests that *Dehalobacter* species dominated the enrichment culture under dechlorination conditions.

Key words: 1,1-dichloroethane; reductive dechlorination; halorespiration; *Dehalobacter*; 16S rRNA gene

CLC number: Q938.1 **Document code**: A

1,1-二氯乙烷微生物降解菌群的生理学鉴定

杨 锐,孙宝林

(合肥微尺度物质科学国家实验室,中国科学技术大学生命科学学院,安徽合肥 230027)

摘要:从纽约哈得逊河河底淤泥样品中富集分离得到的微生物菌群,在厌氧条件下,可将1,1-二氯乙烷通过还原脱氯的呼吸代谢方式降解生成一氯乙烷.这个微生物菌群可以利用氢气、甲酸、乙酸、苯甲酸和延胡索酸作为电子供体进行还原脱氯生长,并能以丙酮酸发酵的呼吸方式生长,但丙酮酸不能用来作为电子供体进行还原脱氯.通过对其他氯代化合物是否能作为电子受体的实验,发现只有1,1-二氯乙烷能被脱氯.在培养基中加入亚硫酸盐可完全抑制二氯乙烷的还原脱氯,但硫酸盐、硝酸盐和延胡索酸盐的加入却不影响还原脱氯.在以氢气作为电子供体进行还原脱氯时,每摩尔氯原子的还原相应地使细胞干重增加(4.402±1.241)g.同时对富集分离的菌群进行16S rRNA基因序列分析发现,在脱氯生长方式下,该微生物菌群中 *Dehalobacter*

Received: 2007-02-12; **Revised**: 2007-05-12

Foundation item: Supported by National Natural Science Foundation of China (30470943).

Biography: YANG Rui, female, born in 1982, master. Research field: microbial ecology. E-mail: yrui@mail.ustc.edu.cn

Corresponding author: SUN Bao-lin, PhD/Prof. E-mail: sunb@ustc.edu.cn

细菌占主导地位.

关键词:1,1-二氯乙烷;还原脱氯;卤代呼吸;*Dehalobacter*;16S rRNA 基因

0 Introduction

Large quantities of chlorinated alkyl hydrocarbons have been released to the environment and many of them have accumulated in groundwater and sediments. Their persistence and toxicity in the environment have posed a great threat to human health and the environment[1]. Studies[2~5] have shown that some halogenated compounds can be reductively dehalogenated by anaerobic bacteria in a halorespiratory process. Reductive dechlorination of chlorinated ethenes (tetrachloroethene (PCE) and trichloroethene (TCE)) has been intensively studied over the last fifteen years[6~11], while relatively less work regarding the degradation of chlorinated ethanes has been reported, despite their widespread distribution in the environment[12]. So far, several bacteria capable of reductively dechlorinating chlorinated ethanes have been isolated and characterized. One of these bacteria is *Dehalobacter* species strain TCA1[13], which reductively dechlorinates 1,1,1-trichloroethane (1, 1, 1-TCA) to chloroethane (CA). 1, 2-dichloroethane (1, 2-DCA) can be degraded by several bacterial strains, such as *Dehalococcoides ethenogenes* strain 195[14], *Dehalococcoides* sp. strain BAV1[15], *Desulfitobacterium dichloroeliminans* strain DCA1[16], and *Dehalobacter* sp. strain WL[17].

1, 1-dichloroethane (DCA) is often used as intermediate organic solvent to make other chemicals like 1, 1, 1-TCA and as degreaser to dissolve and remove grease. Due to inadvertent spilling or inappropriate disposal of industrial wastes containing DCA, it has been found in at least 248 of the 1 430 National Priorities List sites identified by the U. S Environmental Protection Agency. *Dehalobacter* species strain TCA1 can dechlorinate DCA to CA[13]. In the process of reductive dechlorination, detoxification of 1, 1, 1-TCA was sequential with the accumulation of DCA before conversion to CA. In the present study, we describe the characterization of a microbial consortium reductively dechlorinating DCA to CA enriched from the sediment sample of Hudson River, New York. In particular, we found *Dehalobacter* species as dominant populations in the highly enriched culture, further demonstrating the important role of *Dehalobacter* bacteria in polluted anaerobic environments.

1 Materials and methods

1.1 Bacterial enrichment and cultivation

About 10 g sediments from upper Hudson River, New York were mixed under anaerobic conditions with 100 mL of anaerobic synthetic medium in 160 mL serum bottles as described previously[13]. The anaerobic medium was amended with 2.5 mmol/L acetate plus 4.5 mmol/L H_2 as electron donors. DCA (neat, 4 μL) was then injected into each bottle, giving a final concentration of 560 μmol/L. Cultures were incubated in inverted bottles without shaking in the dark at 27 ℃. Autoclaved cultures were used as negative controls. The active microcosms were fed with DCA for two more times before further transfers were made. After about 2 mmol/L DCA was consumed, a serial dilution (10^{-1} to 10^{-8}) was made for further enrichment of DCA dechlorinating populations. 2-bromoethane sulfonic acid (BESA) was added into the serial transfer cultures at a final concentration of 1 mmol/L.

1.2 Phenotypic characterization

To determine the range of electron donors used in highly enriched cultures, we prepared triplicates of 30 mL basal media containing 560 μmol/L DCA in each case with either H_2, formate, acetate, fumarate, glucose, lactate, benzoate or pyruvate as electron donors at 5 mmol/

L. Acetate at 250 μmol/L as carbon source was added when formate and H_2 were tested as electron donors for reductive dechlorination. Reductive dechlorination and growth were determined by measuring the depletion of DCA and the production of CA.

To determine the electron acceptor range, triplicates of 30 mL anaerobic media were amended with 2.5 mmol/L acetate as carbon source and 4.5 mmol/L H_2 as electron donor. Chloroethane (CA), 1, 2-dichloroethane (1, 2-DCA), 1, 1, 1-trichloroethane (1, 1, 1-TCA), 3-chlorobenzoate, 3-Cl-4-OH-phenylacetate (Cl-OHPA), 2-chlorophenol, PCE and TCE were tested as potential electron acceptors at 250 μmol/L. The media were inoculated with 2% (v/v) of active culture and incubated in the dark at 27 ℃. The cultures were periodically monitored for the depletion of electron acceptors and appearance of products.

To determine whether the reductive dechlorination activity was inhibited by other chemicals, duplicate cultures were grown on acetate, H_2 and DCA plus either 5 mmol/L sulfate, sulfite, nitrate or fumarate, respectively. The effect of inhibition was determined by measuring the depletion of DCA and production of CA over three successive feedings and by monitoring with gas chromatography (GC).

To determine whether the consortium could grow under pyruvate fermentation or sulfate-reduction condition, triplicates of 50 mL basal media were amended with 5 mmol/L pyruvate or 2.5 mmol/L sulfate along with 5 mmol/L lactate. To determine the competition capability of various bacterial populations for electron donors, 560 μmol/L DCA was added into the aforementioned cultures. Growth was determined by observing the increase in visual culture turbidity.

1.3 Dechlorination rate and growth yield

Reductive dechlorination was determined by the depletion of DCA and the production of CA. Samples from triplicate cultures were measured every 24 h by GC analysis. The cell yields from reductive dechlorination were determined by dry-weight measurements from duplicate 100 mL cultures. Basal media were amended with 1 mmol/L DCA, plus either 4.5 mmol/L H_2 and 2.5 mmol/L acetate, or 4.5 mmol/L formate and 0.5 mmol/L acetate, or 5 mmol/L acetate, respectively. Control cultures consisted of the same media but without DCA. After DCA was completely dechlorinated to CA, the cells were collected by filtration on pre-weighed 0.22 μm membrane filters. The membranes with cells were transferred onto pre-weighed, micro-size weighing dishes, dried overnight at 40 ℃, and weighed until constant masses were obtained. Growth yields via reductive dechlorination were calculated by subtracting the cell yields of the no DCA amended cultures from the DCA amended cell yields.

1.4 Chemical analysis

DCA, CA, 1, 1, 1-TCA and 1, 2-DCA were analyzed by manually injecting 0.1 mL of headspace gas into GC (Agilent 6 890 N) with a flame ionization detector (FID), a capillary column (DB-1, 30 m×0.25 mm (inner diameter)), a split injection ratio of 10 ∶ 1, and a flow rate of 2.0 mL/min of nitrogen as a carrier gas. The oven temperature program was 50 ℃ for 4.5 min, and then increased to 150 ℃ at 40 ℃/min with a final hold at 150 ℃ for 0.5 min. PCE and TCE were analyzed by GC (Agilent 6 890 N) with an FID detector, a capillary column (DB-1), a split injection ratio of 3 ∶ 1, and a flow rate of 1.5 mL/min of nitrogen as carrier gas. The oven temperature was 50 ℃ for 1.0 min, and then increased to 150 ℃ at 30 ℃/min with a final hold at 150 ℃ for 1.5 min. The temperatures of the injector and detector were 80 and 300 ℃, respectively. Calibration curves were prepared by adding a known amount of the compounds to 160 mL serum bottles containing 50 mL of medium to give the same liquid-to-headspace ratio as that for the cultures. 2-chlorophenol, 3-chlorobenzoate,

Cl-OHPA and their dechlorination products were analyzed by high-performance liquid chromatography (Agilent 1 100 Series) with reverse C_{18} column (Phenomenex) with a flow rate of 1 mL/min, a mobile phase of acetonitrile : water : H_3PO_4 with a ratio of 33 : 66 : 1, a injection volume of 20 μL, and a UV detector set to 218 nm for 2-chlorophenol, 230 nm for 3-chlorobenzoate and 206 nm for Cl-OHPA, respectively. The depletion of chlorinated compounds and appearance of products were quantified by comparison with standard calibration curves and with zero time samples.

1.5 16S rDNA gene cloning and analysis

DNA was extracted by using the Wizard Genomic DNA purification kit (Promega) from 100 mL culture which utilized H_2 as electron donor. The 16S rRNA gene was amplified by using primers FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-AAGGAGGTGATCCAGCC-3′)[18] and a PCR protocol for this purpose[19]. The PCR products (approximate 1 560 bp) were purified by using Wizard PCR Preps DNA purification system (Promega). The purified PCR products were cloned into the PCR$^{2.1}$-TOPO vector transformed into One Shot TOP10 competent cells with TOPO TA Cloning Kit (Invitrogen). Six positive colonies were randomly picked for sequencing using M13f (5′-GTAAAACGACGGCCAG-3′) and M13r (5′-CAGGAAACAGCTATGAC-3′) with automated fluorescent dye terminator sequencer (Invitrogen Company, Model 3730 Sequenator). The resulting sequences were analyzed and the phylogenetic placement was obtained using RDP Ⅱ (Ribosome Database Project Ⅱ) and BLAST (Basic Local Alignment Search Tool). A Neighbor-Joining phylogenetic tree was created by software Mega 3.1.

1.6 GenBank accession number

The 16S rRNA gene nucleotide sequence determined in this study has been deposited into the GenBank database under Accession No. DQ777749 for *Dehalobacter* species 1,1-DCA1.

2 Results and discussion

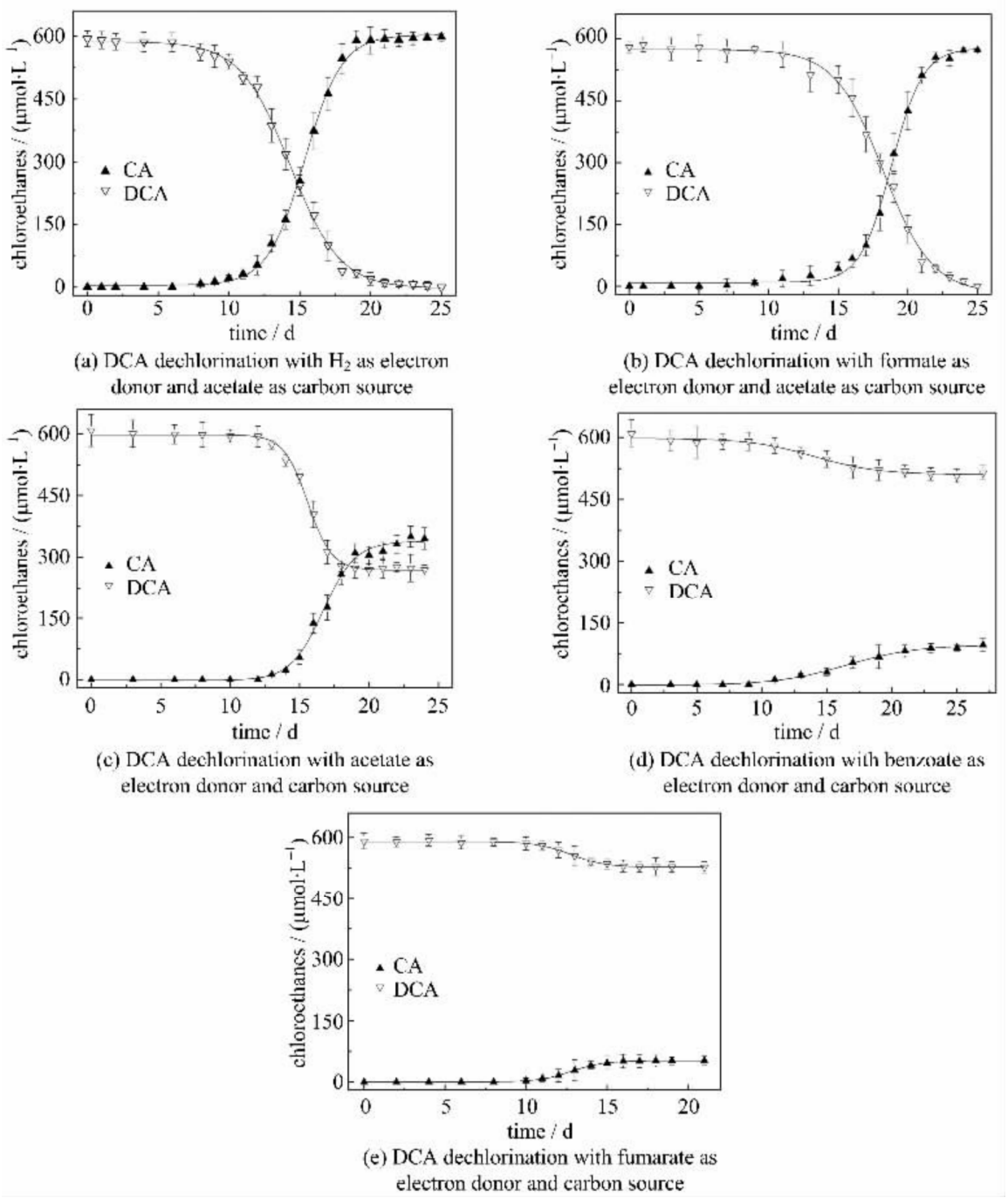
2.1 Enrichment of DCA-dechlorinating culture

Microcosms established with Hudson River sediment sample reductively dechlorinated DCA after 2 months of incubation. The enrichment was able to completely convert DCA to CA in the temperature range of 20～30 ℃. No dechlorination was observed in autoclaved cultures. Initially, active methanogenesis was observed in dechlorinating micocosms since large amounts of methane was detected. Therefore, a methanogenic inhibitor, BESA, was added into serial transfer cultures. The addition of BESA in subsequent transfers led to the loss of methanogenesis, but the dechlorination activity was not affected, indicating that DCA dechlorination in these cultures was not cometabolic with methanogenesis.

DCA dechlorination gave rise to CA as the final product in our enrichment cultures (Fig. 1). Previous studies have shown that DCA and CA can be cometabolized in the aerobic environment, but DCA transformation under aerobic conditions is much slower than that under anaerobic conditions[12,20]. Since aerobic transformation of DCA is slower than that of CA, complete DCA conversion to CA would result in better feasibility and efficiency for bioremediation of chlorinated ethanes[21].

2.2 Physiological characterization

Among the electron donors tested, the enrichment culture used acetate, H_2, formate, benzoate and fumarate as electron donors for reductive dechlorination. The consortium could not use lactate, pyruvate, or glucose as electron donors for reductive dechlorination although pyruvate fermentation and sulfate reduction with lactate as electron donor occurred. This is a little surprising, since pyruvate fermentation usually produces H_2 as a reductive equivalent, which could serve as electron donor for reductive dechlorination. Subsequent DCA dechlorination was not observed following pyruvate fermentation



Error bars represent standard deviations of triplicate cultures

Fig. 1　Stoichiometry of the reductive dechlorination of DCA to CA by enrichment cultures

and sulfate reduction, suggesting that bacterial populations other than dechlorinators had consumed most of nutrients such as vitamins that are essential to reductive dechlorination and growth.

It seems that both H_2 and formate are favourable electron donors for DCA dechlorination (Fig. 1(a), Fig. 1(b)). The coupling of bacterial growth to DCA dechlorination with H_2 as electron donor was revealed by a growth yield of (4.402±1.241)g of cells (dry weight) per mole of chloride released. Although formate has a much longer lag phase, the dechlorination pattern was similar to that of H_2. The growth yield from DCA dechlorination with formate as electron donor was (4.181±0.381)g of cells (dry weight) per mole of chloride released. Acetate also can be used as electron donor, although the dechlorination rate was much slower (Fig. 1(c)), and a growth yield of (1.197±0.52)g of cells (dry weight) was obtained per mole of chloride released. Benzoate (Fig. 1(d)) and fumarate (Fig. 1(e)) were also used as electron donors for DCA dechlorination, but the dechlorination rates were much slower,

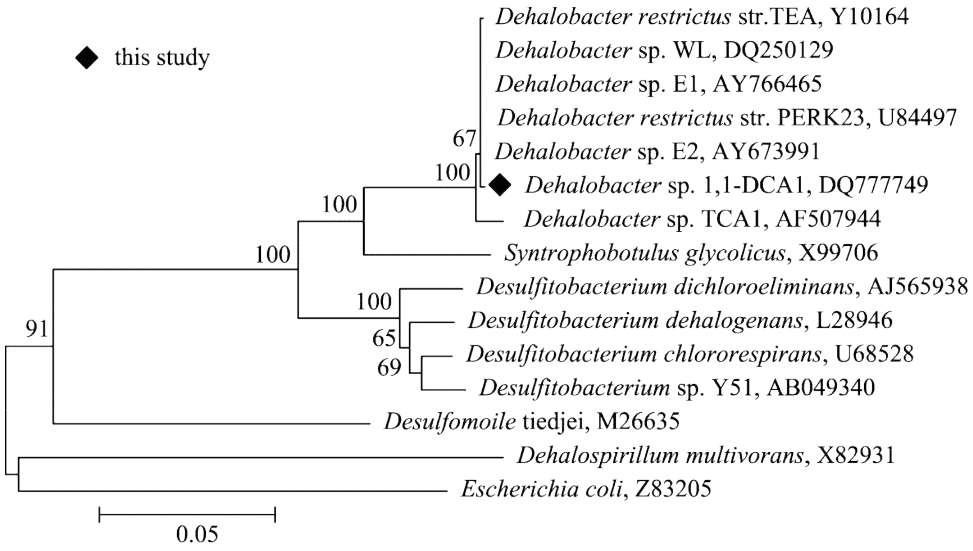
and the growth yields from these cultures could not be detected because of the low biomass. The cell yields were directly proportional to the amounts of DCA dechlorination, implying that DCA dechlorination may occurr in a respiratory process. This growth-linked respiratory process would be more desirable in *in situ* bioremediation.

Among the halogenated compounds tested, only DCA was reductively dechlorinated. The consortium did not dechlorinate 1,1,1-TCA, 1,2-DCA, CA, PCE, TCE, Cl-OHPA, 3-chlorobenzoate, and 2-chlorophenol over a period of 3 months. Among other potential electron acceptors tested, the consortium could only grow under sulfate-reducing conditions. The consortium used lactate as electron donor for sulfate reduction but not DCA dechlorination, suggesting that sulfate reducers and reductive dechlorinators are different bacteria populations in our consortium.

Reductive dechlorination may be inhibited by other chemicals as competitive electron acceptors. In our cultures, 5 mmol/L sulfate, nitrate or fumarate had no effect on DCA dechlorination, whereas 5 mmol/L sulfite completely inhibited DCA dechlorination. Since most reductive dehalogenases and related components such as electron carriers are membrane bound, it is possible that DCA dechlorination and sulfite reduction share some parts of the electron transport chain.

2.3 Phylogenetic analysis

To determine the phylogenetic placement of the dominant populations responsible for DCA dechlorination, 16S rRNA genes of the consortium were cloned and randomly sequenced. The sequences of six clones were selected and compared with already known sequences in RDP-Ⅱ. All these sequences fell into the subphylum of the gram-positive bacteria with low G + C content. Its closest relatives are obligate dechlorinators *Dehalobacter* sp. strain E1 and *Dehalobacter* sp. strain WL (Fig. 2), with similarities of 99.6% and 99.4%, implying that dechlorinating populations were dominant in our DCA dechlorinating consortium. Our consortium did not dechlorinate 1,1,1-TCA as *Dehalobacter* sp. strain TCA1, although both were enriched from Hudson River sediment and had similar growth yields through reductive dechlorination of chloroethanes. On the base of previous studies, all *Dehalobacter* members belong to obligate dechlorinators that only grow by reductive dechlorination of specific chlorinated



Analysis was bootstrapped and bootstrap values greater than 60 are indicated at respective nodes (1000 replications). The scale indicates the number of amino acid substitutions per site

Fig. 2 Unrooted Neighbor-Joining phylogenetic tree based on the 16S rRNA gene sequences amplified from our microbial consortium and representative bacteria from the GenBank database (Accession Numbers are indicated next to the bacterial name)

ethanes or ethenes in anaerobic respiration. Our study further demonstrates the important function and role of *Dehalobacter* bacteria in polluted anaerobic environments.

References

[1] Struijs J, Rogers J E. Reductive dehalogenation of dichloroanilines by anaerobic microorganisms in fresh and dichlorophenol-acclimated pond sediment[J]. Appl Environ Microbiol, 1989,55:2 527-2 531.

[2] Bouchard B, Beaudet R, Villemur R, et al. Isolation and characterization of *Desulfitobacterium frappieri* sp. nov.: An anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-Chlorophenol[J]. International Journal of Systematic Bacteriology, 1996, 46:1 010-1 015.

[3] Distefano T D, Gossett J M, Zinder S H. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis[J]. Appl Environ Microbiol, 1991,57:2 287-2 292.

[4] Schlotelburg C, Von Wintzingerode F, Hauck R, et al. Bacteria of an anaerobic 1, 2-dichloropropane-dechlorinating mixed culture are phylogenetically related to those of other anaerobic dechlorinating consortia[J]. Int J Syst Evol Microbiol, 2000,50(4): 1 505-1 511.

[5] Shelton D R, Tiedje J M. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid[J]. Appl Environ Microbiol, 1984,48:840-848.

[6] Aulenta F, Majone M, Verbo P, et al. Complete dechlorination of tetrachloroethene to ethene in presence of methanogenesis and acetogenesis by an anaerobic sediment microcosm[J]. Biodegradation, 2002,13:411-424.

[7] Damborsky J. Tetrachloroethene-dehalogenating bacteria[J]. Folia Microbiol (Praha), 1999, 44: 247-262.

[8] He J, Sung Y, Krajmalnik-Brown R, et al. Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1, 2-dichloroethene-respiring anaerobe[J]. Environ Microbiol, 2005, 7: 1 442-1 450.

[9] Holliger C, Hahn D, Harmsen H, et al. *Dehalobacter* restrictus gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration[J]. Arch Microbiol, 1998,169:313-321.

[10] Magnuson J K, Stern R V, Gossett J M, et al. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway[J]. Appl Environ Microbiol, 1998,64:1 270-1 275.

[11] Maymo-Gatell X, Tandoi V, Gossett J M, et al. Characterization of an H_2-utilizing enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis[J]. Appl Environ Microbiol, 1995, 61:3 928-3 933.

[12] Mohn W W, Tiedje J M. Microbial reductive dehalogenation[J]. Microbiol Rev, 1992,56:482-507.

[13] Sun B, Griffin B M, Ayala-del-Rio H L, et al. Microbial dehalorespiration with 1,1,1-trichloroethane [J]. Science, 2002,298:1 023-1 025.

[14] Maymo-Gatell X, Chien Y, Gossett J M, et al. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene[J]. Science, 1997, 276: 1 568-1 571.

[15] He J, Ritalahti K M, Aiello M R, et al. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species [J]. Appl Environ Microbiol, 2003,69:996-1 003.

[16] De Wildeman S, Diekert G, Van Langenhove H, et al. Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes[J]. Appl Environ Microbiol, 2003,69:5 643-5 647.

[17] Grostern A, Edwards E A. Growth of *Dehalobacter* and *Dehalococcoides* sp. during degradation of chlorinated ethanes[J]. Appl Environ Microbiol, 2006,72:428-436.

[18] Weisburg W G, Barns S M, Pelletier D A, et al. 16S ribosomal DNA amplification for phylogenetic study [J]. J Bacteriol, 1991,173:697-703.

[19] Holoman T R, Elberson M A, Cutter L A, et al. Characterization of a defined 2, 3, 5, 6-tetrachlorobiphenyl-ortho-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA[J]. Appl Environ Microbiol, 1998,64:3 359-3 367.

[20] Vogel T M, Criddle C S, McCarty P L. Transformations of halogenated aliphatic compounds [J]. Environ Sci Technol, 1987,21:722-736.

[21] Oldenhurs R, Vrnk R L J M, Janssen D B, et al. Degradation of chlorinated aliphatic hydrocarbons by methylosinus trichosporium OB3b expressing soluble methane monooxygenase[J]. Appl Environ Microbiol, 1989,55:2 819-2 826.