Isolation and Characterization of Bacteria that Degrade Poly (Lactic Acid-Glycerol Ester)-Type Time-Release Electron Donors for Accelerated Biological Reductive Dechlorination

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Summary: Hydrogen Release Compound (HRC™) is an important electron donor that has recently become available and is now becoming widely applied to the accelerated biological reductive dechlorination of chloroethenes such as tetrachloroethene (PCE) and trichloroethene (TCE). HRC is a benign poly(lactic acid-glycerol ester) specially formulated for the slow time-release of lactic acid. Lactic acid is then metabolized to hydrogen, which can be used in the reductive dechlorination of chloroethenes. To establish an advance diagnosis of the HRC addition effect for the bioremediation of polluted sites, 17 strains of HRC-degrading bacteria were isolated by liquid- and plate-culture methods. All these strains could grow on a basal medium containing purified HRC as the sole carbon source. The sequence analysis of the 16S rDNAs of 6 of the 17 strains shows that they all belong to the family β-Proteobacteria, which includes *Burkholderia cepacia*, *Burkholderia vietnamiensis*, *Ralstonia* sp., and *Variovorax paradoxus*. The time course of HRC degradaton by strains JM-11, JM-12 and JM-13 showed that the HRC degradation rates after 9 days of cultivation were 81.1%, 82.8% and 80.4%, respectively. Preliminary assay of the activities of the HRC-degrading enzyme indicated that HRC degradation may be specifically performed by specific lipases produced by HRC-degrading microorganisms.

Keywords: degradation; HRC-degrading bacteria; hydrogen release compound (HRC); poly(lactic acid-glycerol ester); strain

Introduction

Tetrachloroethene [perchloroethylene (PCE)] and trichloroethene (TCE) have been widely used as solvents for various industrial processes such as dry cleaning, degreasing and paint stripping. Although these solvents are volatile, the contamination of soil and groundwater

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by PCE and TCE have been widespread because of their high specific gravities. Thus, the remediation of these solvents is now a formidable problem. To solve this problem, *in situ* bioremediation of such contaminants has recently been considered as a favorable, convenient and low-cost process.[1]

Since the 1980s, various microbial degradations and degradation (dechlorination) pathways of PCE and TCE have been studied and clarified. Under aerobic conditions, aerobic cometabolism is used for the remediation of these solvents.[2,3] For cometabolism, in addition to the delivery of oxygen, a cosubstrate is added to induce the production of oxygenases. The cosubstrates include methane, propane, butane, toluene, phenol, or other aromatic hydrocarbons that have been successfully used in supporting TCE degradation. Oxygenases are responsible for the epoxidation and subsequent destruction of contaminants such as dichloroethenes (DCEs) and TCE. Although they are sometimes effective for the treatment of TCE, enhanced aerobic strategies require large amounts of cosubstrate and are not effective against extremely chlorinated compounds such as PCE. For this reason, anaerobic biodegradation has been developed.

Under anaerobic conditions, reductive anaerobic biodegradation is more effective for chloroethene bioremediation. In reductive systems, even highly chlorinated contaminants (e.g., PCE) can be used as electron acceptors, and the biodegradation process begins by supplying an excess of a reduced substrate (electron donor) to a microbial consortium, i.e., a cooperative community of microbial species. Consequently, the selection of an appropriate electron donor may be the key to the development of a population of dechlorinating microorganisms. Recent studies have indicated a prominent role of molecular hydrogen (H) in the reductive dechlorination of chloroethenes. [4-6] Results from such studies suggest that there is competition for hydrogen between reductive dechlorinators and methanogens,[7] i.e., high hydrogen concentrations (saturation up to approximately 80 nM) may favor methanogenic activity, whereas reductive dechlorinators are best supported at much lower hydrogen concentrations (2-10 nM). Therefore, although simple molecules like short chain organic acids and alcohols can be fermented to produce molecular hydrogen, they cannot sustain an effect longer on chloroethenes for dechlorination, since they are fermented to produce hydrogen within a very short time, and the produced hydrogen of high concentration is principally utilized by methanogens for growth.

For this reason, for an ideal approach to reductive dechlorination, an important electron

donor that has just recently become available and is now becoming widely applied is Hydrogen Release Compound (HRC). HRC is a benign poly(lactic acid-glycerol ester) specially formulated for the slow time-release of lactic acid; upon being deposited into the subsurface, it slowly degrades to lactic acid. Lactic acid is then metabolized to hydrogen, which can be used in reductive dechlorination. HRC is a semi-solid material that will remain where emplaced and generate highly diffusable hydrogen slowly over time. Since PCE and TCE plumes are difficult to locate, a continuous and persistent highly diffusable hydrogen source increases the effectiveness of contact, containment and remediation. Also the continuous hydrogen source provided by HRC can reduce dissolved phase PCE and TCE concentrations. This creates a larger concentration gradient which in turn facilitates desorption of PCE and TCE from the soil matrix. Additionally, a low concentration of hydrogen provided by HRC favors the reductive dechlorinators and starves out the methanogens. This has been demonstrated effectively in the laboratory and in the field.[8-11] However, problems in HRC utilization are that contaminated sites resistant to HRC also exist and there is currently insufficient knowledge of the behavior and function of HRC. Bioremediation with HRC is a multistep and slow hydrogen-release process, depending on the size and nature of the microbial population in the vicinity of the application. Farone et al.[12] considered that three groups of microbial populations may associated with dechlorination. Group B1 comprises microorganisms that metabolize PCE and TCE (e.g., *Dehalococoides ethenogenes*);[13] group B2 comprises microorganisms that produce hydrogen (H) from organic acids (i.e., H-producing bacteria); group B3 comprises microorganisms that degrade HRC. However, it is not clear yet which microorganisms are concerned in dechlorination using HRC technology. In fact, there has been no report yet regarding the dechlorination ability of group B3, and the existence of this group is also not yet even clear. The goal of this study is not only to clarify the mechanism of the bioremediation of chloroethenes by HRC utilization, but also to establish the technique for an advance diagnosis of the HRC addition effect for the bioremediation of polluted sites. As a first step to attaining this objective, we isolated and characterized bacteria that could degrade HRC.

Materials and Methods

Materials. HRC manufactured by Regenesis Co., Ltd. (California, USA) was kindly provided by Kokusai Kogyo Co., Ltd (Tokyo, Japan). All the other reagents used were

commercial products of the highest grade available.

Purification of commercial HRC. Since commercial HRC contains a considerable quantity of monomer components, its polymer components were purified from HRC to screen HRC-degrading bacteria. The purification of HRC was performed by solid-phase extraction using a Sep-Pak plus C18 (360 mg) cartridge (Waters Co. Ltd., Milford, USA). Commercial HRC (50 g) was dissolved in 1 liter of distilled water, and then centrifuged at 10,000 rpm for 15 min to separate insoluble components. The precipitate containing insoluble components was washed with distilled water 3 times by centrifugation, and then dissolved in chloroform. The supernatant was loaded onto a C18 Cartridge to remove monomer components. After loading, the retained analyte was washed with distilled water and eluted with chloroform. The eluent and the precipitate dissolved in chloroform were mixed and evaporated at room temperature. The remnant was dissolved in chloroform again and designated as purified HRC.

Media. The composition of the basal medium was as follows (mg $\cdot L^{-1}$): KH2PO4, 450; K2HPO4, 1170; NH4NO3, 1000; MgSO4·7H2O, 100; FeSO4·7H2O, 10; ZnSO4·7H2O, 1; MnSO4·4-6H2O, 2; and CuSO4·7H2O, 0.1. The final pH was adjusted to 10 with 6 M KOH. For the 3-Morpholinopropanesulfonic acid (MOPS) medium, 2000 mg $\cdot L^{-1}$ MOPS was added, and for the solid medium, 15 g$\cdot L^{-1}$ agar was added.

Enrichment of HRC degrading bacteria.

Liquid medium. Groundwater samples collected from TCE-contaminated areas in Japan and soil samples collected from Tsukuba City in Japan were used to screen HRC-degrading bacteria. Purified HRC (final concentration of 2 g $\cdot L^{-1}$) dissolved in chloroform was placed in sterilized test tubes (28 mm diameter), then chloroform was evaporated in a clean bench for 6 hours. After 10 mL of sterilized basal medium or MOPS medium was added to the test tubes, the groundwater (0.4 mL) or soil (0.2 g) samples were also added as a screening source. These test tubes were then incubated at 30°C with shaking (240 rpm). After one week, 0.2 mL of each culture broth was transferred into a new test tube containing fresh medium and purified HRC. This procedure was repeated at least four times. Single-colony isolation from positive cultures was performed using nutrient agar plates, and the isolates were then stored in 200 g $\cdot L^{-1}$ glycerol at −80°C.

Enrichment with solid medium. A filter paper (47 mm diameter) was soaked in acetone solution dissolving purified HRC and acetone was then evaporated in a clean bench. The filter paper was placed on a sterilized petri dish, and 3 mL of the basal medium was

dropped on the filter paper. Then a membrane filter (cellulose acetate, 0.45 μm thickness and 47 mm diameter) soaked with a groundwater sample was placed on the filter paper. After incubation at 30°C for 1 week, colonies that appeared on the membrane filter were isolated using nutrient agar plates and the isolates were then stored in 200 g $\cdot$liter^{-1} glycerol at −80°C.

HRC degradation assay. To investigate the HRC degradation rate of each isolate, two test tubes (16 mm diameter) containing the basal medium (1.2 mL) and purified HRC (2 g $\cdot L^{-1}$) were prepared for each isolate. After inoculation (initial cell concentration was 0.2 as $O.D._{660}$), the tubes were shaken at 30°C on a rotary shaker (BioScreeningSystem, Marubishi Co., Ltd., Japan) at 600 rpm. Uninoculated test tubes were also prepared as a control. After cultivation, the culture broth of one test tube was centrifuged and the supernatant was collected. The other test tube was subjected to alkaline hydrolysis by adding 5 N NaOH, autoclaving and neutralizing with 5 N H_2SO_4. After centrifugation, the supernatant was also collected. Both supernatants were subjected to HPLC (High-Performance Liquid Chromatography) analysis, and residual HRC was determined by measuring the difference between the amounts of hydrolyzed and unhydrolyzed HRC by comparing the production of free lactic acid.

For HPLC, a BioRad Aminex HPX-87H column (7.8×300 mm; BioRad, Munich, Germany) was used, and the column temperature was 50°C. The eluent used was 5 mM H_2SO_4 at a flow rate of 0.5 mL/min. Lactic acid was detected using a refractive index (RI) detector (model YRD-880, Shimamura Keiki Co., Tokyo). The experiments were performed in duplicate.

Bacterial identification. The isolated HRC-degrading bacteria were characterized and identified according to Bergey and Krieng.[13] Strains JM-11, JM-12 and JM-13 of the highest HRC-degrading group, and strains JF-5, JF-8 and JF-10 of different colony shapes were selected, and subjected to phylogenetic 16S rDNA sequence analysis.[14-19]

Amplification of 16S rDNA was performed by direct polymerase chain reaction (PCR), using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1494R (5'-TGACTGAC TGAGGYTACCTTGTTAC-3'). PCR products were excised from a 1.5% agarose gel and purified by BIO 101® systems III GENECLEAN Kit (Qbiogene). Purified PCR products were cloned into a pGEM-T easy vector (Promega) to transform *Escherichia coli* DH10B. Transformation of this clone library was randomly selected for extraction of the recombinant plasmids, using a QIAprep Spin Miniprep Kit (QIAGEN

GMBH). The 16s rDNA sequences of the inserts in the *E.coli* clones were determined using a Model 301 (Applied Biosystem) automated DNA sequencer. Sequences retrieved from 16S rDNA libraries were compared to the 16S rDNA sequences available in the GenBank database using the BLAST program. Sequence was aligned using the multiple sequence alignment software CLUSTAL X, and phylogenetic analysis was performed using the neighbor-joining method.

Time course of cell growth and HRC degradation. Strains JM-11, JM-12 and JM-13 were used to investigate the time course of cell growth and purified-HRC degradation. The procedure was performed according to the stated HRC degradation assay.

Preliminary assay for HRC-degrading enzyme.

Preparation of crude enzyme. To investigate the HRC-degrading enzyme activity, strain JM-13 was chosen due to its HRC-degrading activity. A 500-mL Erlenmeyer flask containing purified HRC (final concentration of approximately 2 g $\cdot L^{-1}$) was placed in a clean bench for several hours to volatilize chloroform. After adding 50 mL of basal medium and inoculating the strain (initial cell concentration of 0.4 at O.D. $_{660}$), the culture was shaken on a rotary shaker at 30°C and 120 rpm for 40 hours, and the culture broth was centrifuged at 10,000 rpm for 20 min to separate the cells from the supernatant. The culture supernatant was concentrated by ultrafiltration, and designated the supernatant concentrate. After adding 20 mM potassium phosphate buffer (pH 7.0) with the same volume of the culture supernatant, the cells were disrupted by sonication, and designated the cell-free extract. The supernatant concentrate and the cell-free extract were both used in assaying the activity of the HRC-degrading enzyme.

Enzyme activity assay. Lipase activity of the supernatant concentrate and the cell-free extract was assayed using Lipase Kit S (Maruko Seiyaku Co., Ltd, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol of free fatty acid per minute. To investigate the degradation of HRC by the crude enzyme, two test tubes, to which purified HRC (final concentration of approximately 2 g $\cdot L^{-1}$) dissolved in chloroform was added, were placed in a clean bench for 4 - 6 hours to volatilize the chloroform. After adding the crude enzymes of the supernatant concentrate and cell-free extract (1.2 mL), the sample in one test tube was subjected to HPLC to measure the amount of free lactic acid at 0 hours, and that in the other tube was incubated at 30°C to induce enzymatic reaction. After reacting for 24 hours, the sample was also subjected to HPLC. HRC degradation by the crude enzyme was represented by an increase

in the amount of free lactic acid within 24 hours. A reaction sample without the crude enzyme was prepared as a control and the experiment was performed in triplicate.

Results and Discussion

Screening of HRC-degrading bacteri. Approximately 400 soil and groundwater samples, which were collected from sites contaminated by TCE, or noncontaminated sites in Japan were screened for HRC-degrading microorganisms using liquid- and plate-culture methods. As a result, 17 strains of HRC-degrading bacteria were successfully isolated. Three strains (named the JK series) were isolated from the basal medium culture, 9 strains (JM series) were isolated from the MOPS medium culture, and 6 strains (JF series) from the plate culture. All these strains could grow on a basal medium containing purified HRC as the sole carbon source. However, there were large differences in cell growth and HRC degradation rates between the strains. Table 1 shows the cell growth ($O.D._{660}$) and the amount of residual HRC after cultivation for 3 and 7 days. The JM-11, JM-12 and JM-13 strains showed higher HRC degradation rates.

Table1. Cell growth and HRC degradation by HRC-degrading isolates.

	Strain	3day		7day	
		O.D.660	Residual HRC (g/l)	O.D.660	Residual HRC (g/l)
	Control	0	1.670	0	1.336
1	JK-1	1.06	0.608	1.42	0.386
2	JK-8	0.79	0.394	0.68	0.344
3	JK-9	1.20	0.363	1.62	0.314
4	JM-1	1.11	0.320	1.30	0.302
5	JM-4	0.48	0.617	0.78	0.391
6	JM-6	0.84	1.279	1.46	0.628
7	JM-7	1.06	0.362	1.24	0.336
8	JM-10	1.12	0.358	1.16	0.262
9	JM-11	1.34	0.426	1.24	0.251
10	JM-12	1.26	0.372	1.40	0.214
11	JM-13	0.96	0.516	0.92	0.135
12	JM-14	1.15	0.335	1.42	0.268
13	JF-5	0.91	0.379	1.00	0.275
14	JF-8	1.10	0.558	1.14	0.386
15	JF-9	1.12	0.302	1.06	0.309
16	JF-10	0.76	1.192	0.58	0.427
17	JF-13	0.19	1.008	0.84	0.469

Since only a low HRC degradation rate was observed in the uninoculated control after incubation for 7 days, the HRC degradation by isolates from this study proceeded through a biotic process. In our previous study, lactic acid-utilizing bacteria could not degrade purified HRC (data not shown). This indicates that bacteria isolated from this study are HRC-utilizing bacteria.

Identification of HRC-degrading bacteria. Strains JM-11, JM-12 and JM-13, which had higher HRC degradation rates, and strains JF-5, JF-8 and JF-10, which had distinct colony shapes, were chosen for further study. The physiological properties of these isolates closely resembled each other. They were all gram-negative, rod-shaped (except JM-11, which was a coccus), oxidase-positive, catalase-positive (JM-12, JF-5, JF-8 and JF-10) or -negative (JM-11 and JM-12) bacteria. Sequence analysis of the forward 500 bases of the 16S rDNA of the 6 strains shows that strains JM-11 and JM-13 have 100% and 99% identities, respectively, with typical strains of *Burkholderia cepacia*; strains JM-12 and JF-5 both have 99% identities with typical strains of *Burkholderia vietnamiensis*; strain JF-8 has 99% identity with typical strains of *Ralstonia* sp.; and strain JF-10 has 99% identity with typical strains of *Variovorax paradoxus*. The phylogenetic tree constructed below (Fig. 1) shows that the six strains all belong to β-Proteobacteria.

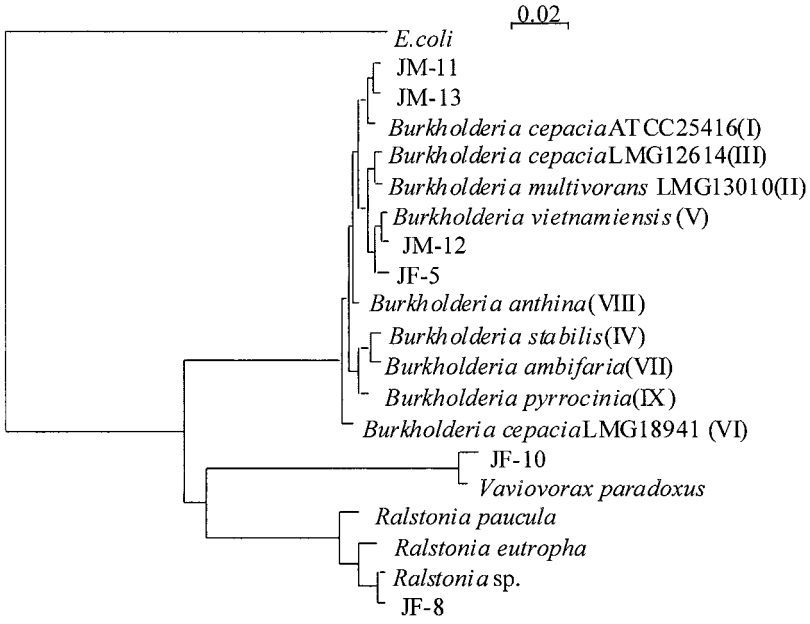

Figure 1. Phylogenetic tree of HRC-degrading bacteria.

Time courses of cell growth and HRC degradation using strains JM-11, JM-12 and JM-13. Strains JM-11, JM-12 and JM-13, which showed high HRC degradation rates, were chosen to determine the time courses of cell growth and purified-HRC degradation. As shown in Fig. 2, the degradation of HRC was accompanied by cell growth. For strains JM-11, JM-12 and JM-13, the HRC degradation rates after 9 days of cultivation were 81.1%, 82.8%, and 80.4%, respectively. Considerable HRC degradation with exponential cell growth was observed for more than 2 days, then moderate HRC degradation and cell growth were observed. The HRC-degrading isolates could also grow on lactic acid as the sole carbon source (data not shown). This seems to suggest that the HRC-degrading isolates primarily utilize monomeric lactic acid as the sole energy source.

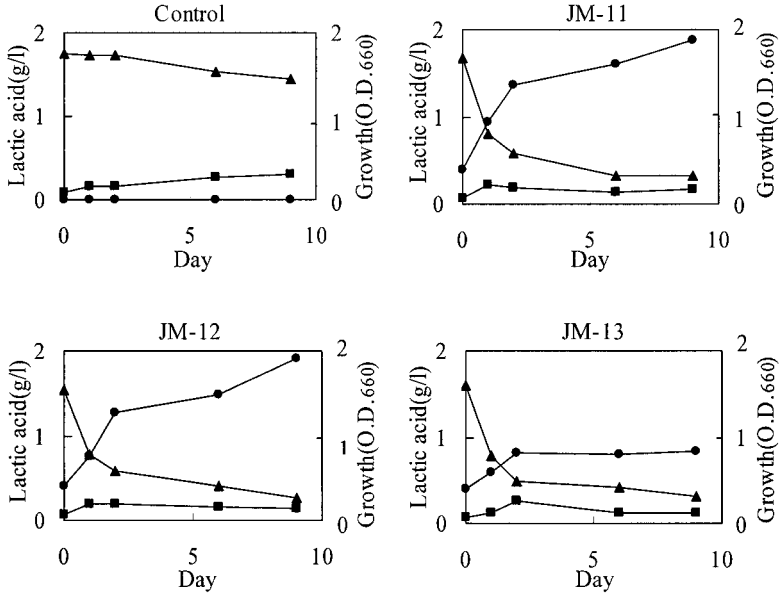

Figure 2. Time courses of HRC degradation by culture broths of strains JM-11, JM-12 and JM-13. HRC degradation was determined as the amount of lactic acid measured by HPLC. Cell growth (●); free lactic acid (■); residual HRC (▲).

Preliminary assay of HRC-degrading enzyme. To investigate the HRC-degrading enzyme, strain JM-13 was chosen because of its high HRC degradation rate. Lipase activity was observed in both the cell-free extract and the supernatant concentrate, and an increase in the amount of lactic acid compared with the control was also observed in the

enzymatic reaction using purified HRC as the substrate (Table 2). The result indicates that strain JM-13 should belong to group B3, which produces lipase for degrading HRC.[12] Thus, strain JM-13 is an HRC-degrading bacteria.

HRC degradation may be specifically performed by specific lipase produced by this strain, since HRC-degrading activity was not detected using the commercial lipase (lipase from *Rhizopus arrhizus*, Boehringer Mannheim GmbH / Germany) (data not shown).

Table 2. Enzymatic assay of strain JM-13.

	HRC degradation (lactic acid increase) (g/l)	Lipase activity (U/l)
Control	0.064	N.D.
Cell-free extract	0.289	12
Supernatant concentrate	0.147	6

N.D.: not detected

Although the HRC supply technique is an efficient approach to *in situ* dechlorination bioremediation, little is known about microorganisms that facilitate reductive dechlororination and the environmental conditions necessary to initiate and sustain HRC activity in contaminated aquifers. A study of the microcosm test using HRC-degrading bacteria is under way in our laboratory.

Conclusions

Bacteria that degrade HRC as an electron donor for accelerated biological reductive dechlorination were isolated and characterized. Seventeen HRC-degrading strains were successfully isolated using the liquid- and plate-culture methods. All these strains could grow on a basal medium containing purified HRC as the sole carbon source. The HRC-degrading isolates could also grow on a basal medium containing lactic acid as the sole carbon source. Strains JM-11, JM-12 and JM-13, which showed higher HRC degradation rates, and strains JF-5, JF-8 and JF-10, which had distinct shape colonies, were chosen for identification. The physiological properties of these isolates closely resembled each other. They were all gram-negative, oxidase-positive, catalase-negative or –positive bacteria. Sequence analysis of the forward 500 bases of the 16S rDNA of the 6 strains shows that strains JM-11 and JM-13 are *Burkholderia cepacia*, strains JM-12 and JF-5 are *Burkholderia vietnamiensis*, strain JF-8 is *Ralstonia* sp, and strain JF-10 is *Variovarax*

paradoxus. In addition, they all belong to the family β-Proteobacteria. The time courses of cell growth and HRC degradation using strains JM-11, JM-12 and JM-13 showed that the HRC degradation rates after 9 days of cultivation are 81.1%, 82.8%, and 80.4%, respectively. Considerable HRC degradation with exponential cell growth was observed for more than 2 days, moderate HRC degradation and cell growth were then observed. This seems to suggest that HRC-degrading isolates primarily utilize monomeric lactic acid as the energy source, and then degrade HRC during their growth. Preliminary assay of the activities of HRC-degrading enzymes indicated that HRC degradation may be specifically performed by specific lipases produced by HRC-degrading microorganisms.

[1] J. l. Sims, J. M. Suflita, H. H. Russell, U.S. Environmental Protection Agency. **1992**, *EPA*/540/S-92/003.
[2] G. D. Hopkins, L. Semprini, P. L. McCarty, *Appl. Environ. Microbiol.* **1993**, *59*, 2277.
[3] M. J. Nelson, S. Montgomery, P. Prichard, *Appl. Environ. Microbiol.* **1988**, *54*, 604.
[4] X. Maymo–Gatell, V. Tandoi, J. M. Gossett, S. H. Zinder, *Appl. Environ. Microbiol.* **1995**, *61*, 3928.
[5] C. Holliger, G. Schraa, A. J. M. Stams, A. J. B. Zehnder, *Appl. Environ. Microbiol.* **1993**, *59*, 2991.
[6] T. D. DiStefano, J. M. Gossett, S. H. Zinder, *Appl. Environ. Microbiol.* **1992**, *58*, 3622.
[7] D. E. Fennell, J. M. Gossett, S. H. Zinder, *Environ. Sci. Technol.***1997**, *31*, 918.
[8] M. Dooley, W. Murray, S. Koenigsberg, A. Leeson, B. C. Alleman (Eds.), Battelle Press, 1999, 121.
[9] S. S. Koenigsberg, C. A. Sandefur, *Remediation Journal* **1999**, *10(1)*,31.
[10] J. K. Sheldon, S. S. Koenigsberg, K. J. Quinn, C. A. Sandefur, A. Leeson and B. C. Alleman, Eds., Battele Press, 1999, 61.
[11] S. Kallur, S. S. Koenigsberg, A. Leeson, B. C. Alleman, Eds., Battelle Press, 1999, 181.
[12] W. A. Farone, S. S. Koenigsberg, J. Hughes, A. Leeson, B. C. Alleman, Eds., Battelle Press, 1999, 287.
[13] G. X. Maymo, Y. Chien, J. M. Gossett, S. H. Zinder, *Science* **1997**, 276, 1568.
[14] D. H. Bergey, Krieng N. R.. "Bergey's Manual of Systematic Bacteriology". Williams & Wilkins Co., 1986, Vol. 2.
[15] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Boil.* **1990**, *215*, 403.
[16] R. I. Amann, W. Ludwig, K. H. Schleifer, *Microbiol. Rev.* **1995**, *59*, 143.
[17] V. Felske, B. Engelen, U. Nubel, H. Backhaus, *Appl. Environ. Microbiol.* **1996**, *62*, 4162.
[18] S. F. Altschul, Stephen F., T. L. Madden, A. A. Schaffer, J. H. Zheng, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **1997**, *25*, 3389.
[19] N. Satio, M. Nei, *Mol. Biol. Envol.* **1987**, *4*, 406.