Isolation and properties of a 2-chlorovinylarsonic acid-degrading microorganism

Kunichika Nakamiya*, Takashi Nakayama, Hiroyasu Ito, Yasuyuki Shibata, Masatoshi Morita

Division of Environmental Chemistry, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

ARTICLE INFO

ABSTRACT

2-Chlorovinylarsonic acid (CVAOA) is a stable abiotic metabolite of lewisite 1 that has been identified in lewisite dumps. There have been no reports of microbial degradation of CVAOA, so we isolated and examined CVAOA-degrading microorganisms. CVAOA contains arsine, which is toxic to microbial growth. We therefore used the simple organic chemical, ethylene, as a sole carbon source in initial screening for suitable microbes. We isolated several microorganisms from sewage sludge and soil. Two strains, NK0505 and NK0506, could be grown on CVAOA as the sole carbon source and were identified by 16S rRNA sequencing as *Nocardia carnea* NK0505 and *Rhodococcus opacus* NK0506. Because *N. carnea* NK0505 was slightly more active in degrading CVAOA, we used it for further degradation studies. Strain NK0505 utilized about 90% of CVAOA (50 ppm) within 5 days; at higher concentrations of CVAOA no degradation occurred over a 10-day period. We identified 1-chloro-1,2-dihydroxyethane, ethylene glycol, glycolic acid, and arsenic acid as degradation products of CVAOA. Epoxy formation on alkylarsine was not confirmed. CVAOA is probably further metabolized via these compounds in the tricarboxylic acid cycle. Strain NK0505 could also degrade *but*-3-enylarsonic acid, trichloroethylene, isoprene, and 1,3-butadiene, but utilization of tetrachloroethylene and acetylene did not occur.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Lewisite is a chemical weapon composed mainly of 2-chlorovinyldichloroarsine (lewisite 1; L-1); it has olefin and arsine in its structure. Two isomers, bis(2-chlorovinyl) chloroarsine (lewisite 2) and tris(2-chlorovinyl) arsine (lewisite 3), are included in lewisite, but L-1 usually accounts for about 80% to 85% of the compound when it has been synthesized from mercury chloride [1]. Skin contact with lewisite causes effects as blisters, and inhalation of lewisite results in death [1]. L-1 is easily hydrolyzed to 2-chlorovinylarsonous acid, 2-chlorovinylarsinic oxide, and 2-chlorovinylarsonic acid (CVAOA), and these compounds are also toxic [1].

There have been no reports confirming the solubility of the concentration limits of CVAOA in water or soil. However, by my observation, synthesized CVAOA is nearly freely soluble in water. CVAOA produced from lewisite hydrolysis has been estimated to have greater water solubility than lewisite, to a concentration limit of 500 mg/L [1]. Furthermore CVAOA is relatively stable than lewisite and other degradation product in water and, like diphenylarsinic acid and phenylarsonic acid, is likely to be capable of spread into the soil and groundwater [2].

There have been several reports of the degradation treatment of lewisite and other chemical warfare agents. Haas et al. [3] attempted to degrade lewisite with the fungal enzyme manganese peroxidase. In 30 min, 98.8% of the lewisite present in reaction mixtures at 34–250 μg/mL had been degraded. This was a fast treatment speed, but the degradation products of this reaction were not described. Boronin et al. [4] attempted to degrade a mustard–lewisite mixture by alkaline hydrolysis and electrochemical treatment; the detoxification products, including bis(2-hydroxyethyl)sulfoxide and other arsenical organic compounds, were treated by fluidized-bed bioreactor.

Microbial degradation of the natural organic arsenic compound arsenobetaine was studied well by Jenkins et al. [5]. Two degradation pathways were proposed, and it was estimated that the methyl or carboxymethyl moieties of this compound were decomposed to produce oxides. However, there have been no reports of either the microbial degradation of CVAOA by single strains of microorganisms or the analysis of the degradation products.

Microbial degradation of olefin-containing organic compounds has been investigated. For example, the natural olefin ethylene is utilized by *Mycobacterium* sp., *Nocardia* sp., and *Xanthobacter* sp. as a sole source of carbon [6]. The organic solvent trichloroethylene (TCE) is degraded under aerobic [7], anaerobic [8], and methanogenic [9] conditions by microorganisms.

CVAOA has been identified in chemical weapons dumps. Siegel [10] suggested that it can persist in seawater for several months

* Corresponding author. Tel.: +81 29 850 2913; fax: +81 29 850 2570.
E-mail address: nakamiya.kunichika@nies.go.jp (K. Nakamiya).

or even longer. There is no serious pollution by CVAOA in Japan, but there is pollution by diphenylarsonic acid and phenylarsonic acid (the deposited starting materials for diphenylchloroarsine and dichlorophenylarsonic acid, respectively) in the cities of Kamisu in Ibaraki Prefecture and Hiratsuka in Kanagawa Prefecture [11]. Diphenylarsonic acid–and phenylarsonic acid–degrading microorganisms have been already isolated [12]. Today, there is concern about the possibility of pollution by CVAOA. We therefore need to determine the environmental fate of CVAOA degraded by microorganisms and to apply microorganisms in the bioremediation of CVAOA, but we have no information on CVAOA-degrading microorganisms. Here, we tried to isolate CVAOA-degrading microorganisms from the soil by using ethylene as the sole source of carbon; we then examined the specificity of a number of organisms in metabolizing CVAOA.

2. Materials and methods

2.1. Chemicals

CVAOA was synthesized by the method of Lewis et al. [13]. 3-Enylarsonic acid and *rac*-3,4-dihydroxybutylarsonic acid were synthesized by the method of Serves et al. [14]. 4-Chloro-2-phenyl-1,3,2-dioxaborolane was synthesized by the method of Wulf and co-workers [15,16]. Other chemicals were of laboratory grade.

2.2. Cultivation conditions and degradation tests

The mineral salts (MS) medium used by De Bont [17] was used to isolate ethylene degradation strains. The pH was adjusted to 6.8 with HCl and/or NaOH. For the preparation of a solid medium, 1.5% agar was added to the MS medium. For the isolation of microorganisms, cotton-plugged vials and/or laboratory dishes containing the medium were placed in a large, sealed container (Anaerobic Jar, Sanshinkogyo, Co. Ltd, Yokohama, Japan) and about 5% of the headspace was replaced by ethylene gas (Japan Fine Products Co. Ltd, Kawasaki, Japan) by syringe. For the degradation test, 3 mL of medium was added to a vial (30 mL). After sterilization of the vial at 121 °C for 10 min and inoculation with the culture medium (0.3 mL), the vial was plugged with a butyl rubber stopper and capped with an aluminum seal; 5% of the headspace was replaced with filtered (Puradisc 25 AS, Whatman, Middlesex, UK) sterilized gas by syringe under sterile conditions. When alkyl arsonic acids or chlorinated ethenes were used as the sole source of carbon, the medium was first sterilized at 121 °C for 10 min and the required concentration of an aqueous solution of alkyl arsonic acids or chlorinated ethenes was added; the microbial isolates were then inoculated into the medium and cultivated at 30 °C with shaking by rotary shaker. Growth was estimated from the protein concentration in the culture media by the Bradford method [18] or by counting the number of colony forming units on Luria-Bertani (LB)-medium. Bovine serum albumin was used as the calibration standard.

2.3. Identification of microbes from 16S and 18S rRNA

The methods used to extract genomic DNA and amplify the 16S rRNA were as described previously [19], with a modification. Hexadecyltrimethylammonium bromide treatment was used for the purification step [20] in the extraction of bacterial genomic DNA. The PCR product was sequenced at Hokkaido System Science Co., Ltd., Hokkaido, Japan. The sequence obtained was identified from the database of the DNA Data Bank of Japan (DDBJ).

2.4. Analysis of organic arsenical compounds

To 0.5 mL of culture medium, we added 25 μL of 2N HCl and 5 μL of 1-propanethiol and then shook the mixture overnight at room temperature; we then extracted it in nonane (for quantification of organic arsenical compounds) or ethylacetic acid (for detection of degradation products; each 200 μL). The nonane phase was dried over anhydrous Na_2SO_4 and analyzed by gas chromatography–mass spectrometry (GC–MS) (GC-mate mass spectrometer, JEOL Ltd., Tokyo, Japan; HP 6890 gas chromatograph, Agilent Technologies, Santa Clara, CA, USA). The ethylacetic acid phase was dried under N_2 flow at room temperature and then redissolved in 50 μL of ethylacetate and analyzed by GC–MS.

2.5. Analysis of dihydroxyl residues containing organic acids

One volume of culture medium was mixed with an equal volume of methanol and centrifuged for 5 min (12,000 rpm) at room temperature. The supernatant was removed and an equal volume of 80 mM methanolic phenylboronate was added to derivatize the degradation products [21]. The mixture was shaken for 5 min at room temperature and then dried by the addition of anhydrous Na_2SO_4.

For those samples used to look for complex degradation products on the GC–MS chromatograph, the ethyl acetate phase from acidified culture medium was dried (Na_2SO_4) and treated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, 1 + 4, v/v) for 1 h at room temperature.

The dried sample was analyzed by GC–MS. Separations were performed on a DB-17MS column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA, USA), and the analytes were ionized in electron impact mode. The oven temperature program was 80 °C (hold for 2 min), 150 °C (20 °C/min), and 300 °C (10 °C/min).

2.6. Analysis of volatile olefins

After cultivation, the culture medium was boiled in a water bath for 30 min and the olefin vapor in the head space was analyzed by GC–MS as described above. Separations were performed on a DB-Wax column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA, USA).

3. Results and discussion

3.1. Isolation of CVAOA-degrading microorganisms by using ethylene as the sole source of carbon on first screening

We had previously tried to isolate CVAOA-degrading microorganisms from sewage samples and soil. However, we were not successful in isolating microorganisms by using CVAOA as the sole source of carbon; it seemed that the toxicity of CVAOA – especially of the arsine in its structure – was preventing the growth of the microbes. Because the chlorovinyl moiety seemed likely to be easily degraded by microorganisms, as a first step in the isolation of microorganisms we selected out ethylene-degrading microorganisms. We isolated several microbes by using ethylene as the sole source of carbon. We then cultivated these microbes on CVAOA at various concentrations as the sole source of carbon. From this selection we found two isolates, strains NK0505 and NK0506 (Fig. 1). Strains NK0505 and NK0506 were identified respectively as *Nocardia carnea* and *Rhodococcus opacus* by 16S rRNA sequencing. The accession number of *N. carnea* NK0505 was AB252192 and that of *R. opacus* NK0506 was AB252191.
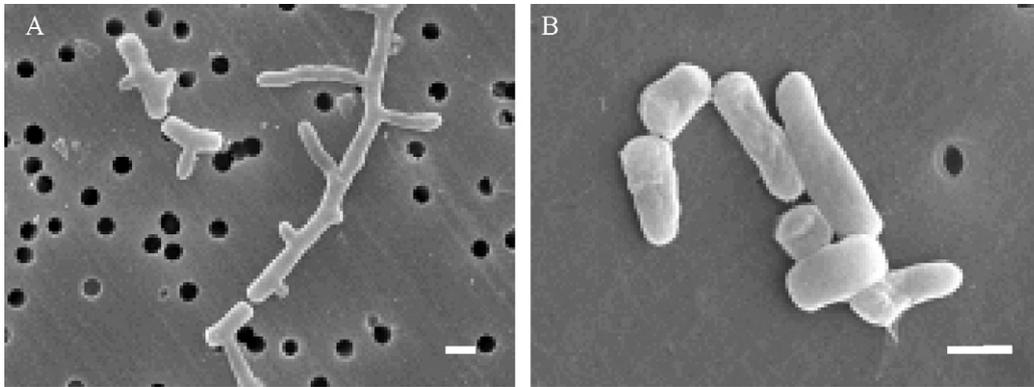
Fig. 1. Scanning electron micrographs of isolated microorganisms. Bar, 1 μm. (A) Strain NK0505; (B) Strain NK0506.

3.2. Effect of CVAOA on the growth of strains and substrate specificity of the isolate N. carnea NK0505

The effects of changes in the concentration of CVAOA on degradation by both strains were almost equal (Fig. 2A). Both strains could not grow at CVAOA concentrations over 1 g/L during cultivation for 10 days (data not shown). Inhibition of microbial growth may have been caused by the toxicity of CVAOA to the bacteria. Strain NK0505 grew slightly faster than NK0506; we therefore used *N. carnea* NK0505 for further studies of the degradation of CVAOA. Strain NK0505 utilized about 10% of CVAOA at an initial concentration of 500 mg/L within 5 days (Fig. 2B), but degradation stopped after 30 h of cultivation. However, more than 90% of CVAOA at an initial concentration of 50 mg/L was utilized over 5 days. These results indicated that the degradation product As(V) hindered the growth of strain NK0505; its concentration was estimated to be about 50 mg/L (10% of CVAOA at an initial concentration 500 mg/L) (equivalent to As 20 mg/L).

The solubility of lewisite (M.W. 207.3) in water is 500 mg/L [1]. When lewisite leaks at this concentration into groundwater, CVAOA (M.W. 186.4) at about 450 mg/L will be produced. CVAOA will be diluted in water, its concentration maybe not increased over 450 mg/L by limitation of water solubility of lewisite. If strain NK0505 were to be used in bioremediation, this strain would not effectively degrade CVAOA present in dumps at near area. However, if it were to work at sites where there were away from dumps of lewisite, because the concentration of the degradation product As(V) would decrease to about 20 mg/L. The national standard for arsine in ground water in Japan is 0.01 mg/L; as elemental 20 mg/L of As(V) is still enough toxic for environment. The use of strain NK0505 for bioremediation would be limited to areas of low CVAOA concentration unless an additional method to adsorb As(V) were to be used.

When more than 20 mg/L of arsenic acid without CVAOA was used for the cultivation of strain NK0505 under ethylene gas, growth of strain NK0505 was not inhibited (data not shown). An inhibitory effect was not seen until the arsenic acid concentration exceeded and reached 1000 mg/L. These results indicated that strain NK0505 could grow in the presence of excess carbon, and that any defense effect for arsenic acid occurred. In this case methylation of arsenic acid and cumulative disappearance of the arsenic acid from the culture medium were not observed. One of the possible biochemical explanations is that ATPase is activated and there is exocytosis of toxic materials, with accelerated removal of arsenic acid from the bacterial cells. These results suggest that, with supplemented nutrition, NK0505 could use CVAOA at higher concentrations at contaminated sites and could be used in bioreactors for the treatment of lewisite. However, more studies are needed to explain the ability of NK0505 to grown in the presence of high concentrations of arsenic acid.
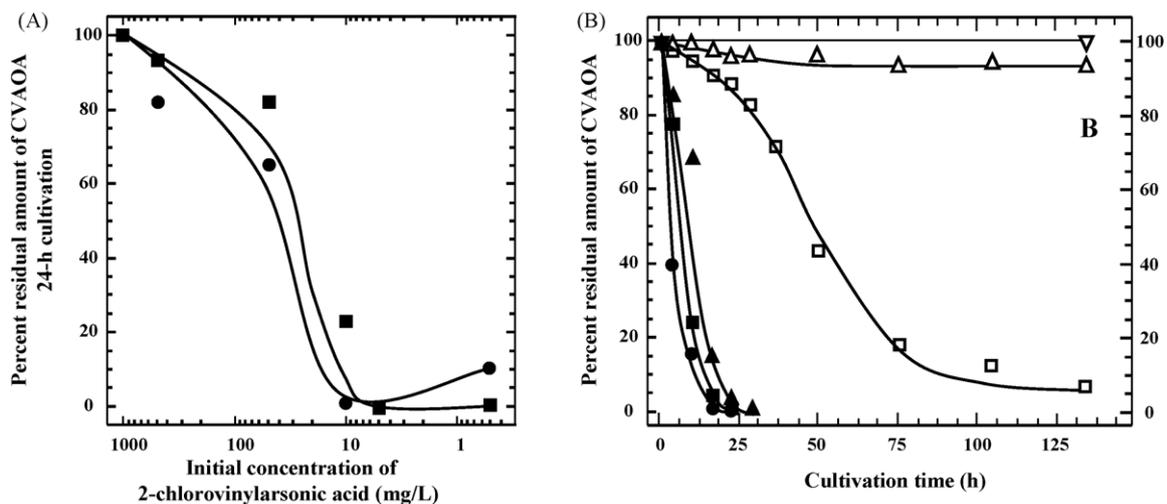
Fig. 2. Effect of substrate concentration on degradation of CVAOA (A); and time course of degradation of CVAOA at various initial concentrations (B). (A) Closed circles, strain NK0505; closed squares, strain NK0506. (B) Degradation by strain NK0505. Closed circles, CVAOA 0.5 mg/L; closed squares, 5 mg/L; closed triangles, 10 mg/L; open squares, 50 mg/L; open triangles, 500 mg/L; open inverted triangles, blank (not inoculated).
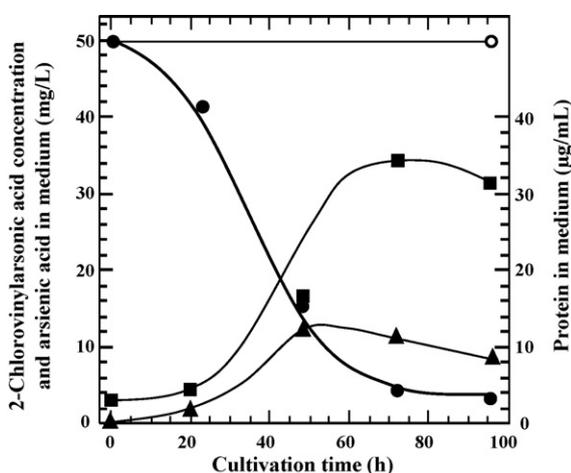
Fig. 3. Time course of degradation of CVAOA by strain NK0505. Closed circles, CVAOA concentration in medium (mg/L); closed triangles, arsenic acid (mg/L); closed squares, protein in medium (μg/mL). Each point is the mean of three replicates.

NK0505 could degrade and/or utilize *but*-3-enylarsonic acid, TCE, isoprene, and 1,3-butadiene, but utilization of tetrachloroethylene (perchloroethylene; PCE) and acetylene was not observed (Table 1). Arsenic acid was present on GC–MS of samples from the degraded medium (data not shown). Our isolate could utilize some olefins (but not PCE) as a growth substrate. Usually PCE is not utilized by aerobic microorganisms because it is oxidized by four chloride atoms and enzymes cannot attack the core structure; however, under anaerobic conditions microorganisms can degrade it by reductively removing the chloride [8]. *rac*-3,4-Dihydroxybutylarsonic acid was detected as the degradation product of *but*-3-enylarsonic acid. This result indicated that degradation of the olefin moiety of CVAOA was proceeded by the hydroxylation of double bonds. *Nocardia rhodochrous* can utilize acetylene gas [22], but our isolate did not utilize it. Rosner et al. [23] and Germon et al. [24] reported the utilization of acetylene by *Rhodococcus ruber* and *Rhodococcus rhodochrous* E5, respectively, but our other isolate, *R. opacus* NK0506, did not utilize acetylene as a sole carbon source (data not shown). Usually nitrogenase can degrade acetylene to ethylene, and it has been applied to the analysis of nitrogenase activity on root nodules. Probably our isolate had weak nitrogenase activity or only a small amount of nitrogenase.

The time course of the growth of strain NK0505 in medium containing 50 mg/L CVAOA is shown in Fig. 3. The decrease of CVAOA in the medium was essentially complete after 5 days, and there was a concomitant increase in the concentration of the degradation product arsenic acid (about 12 mg/L after 2 days) and protein (35 μg/mL).

3.3. Identification of degradation products of CVAOA

We proposed a degradation pathway for CVAOA. There was a small peak with a parent mass of 303.0 (Retention time 11.55; equal to the propanethiol derivative of epoxy-forming CVAOA) on the slightly late retention time of the GC–MS chromatograph
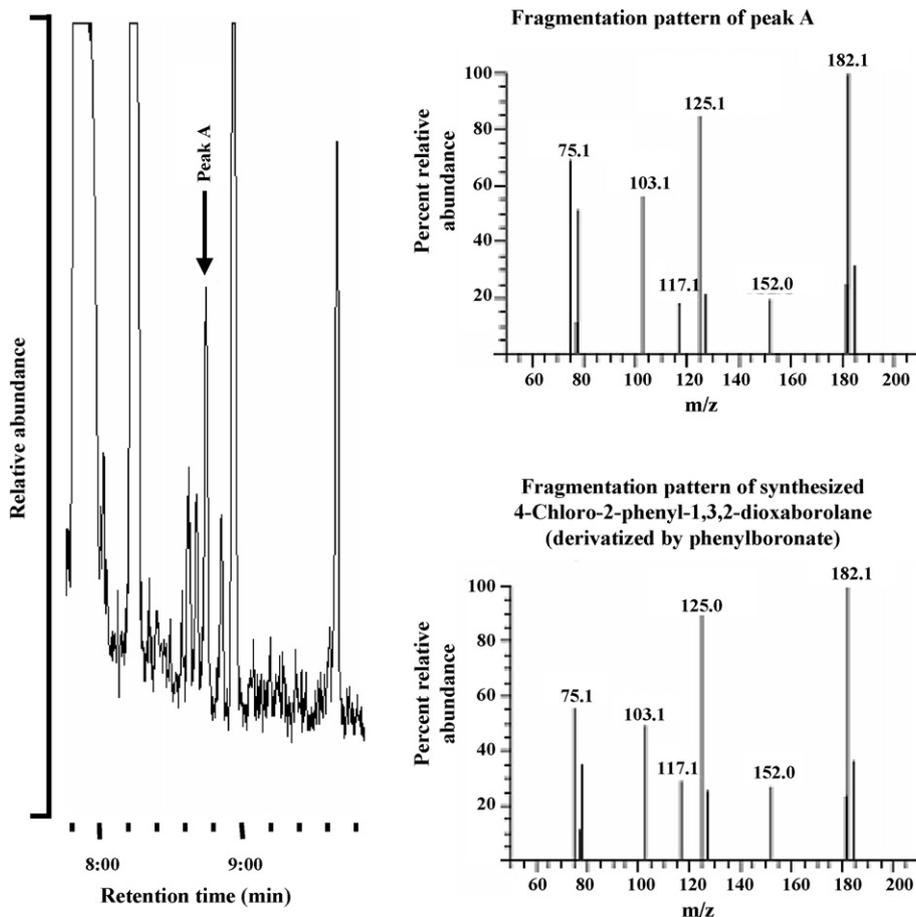
Fig. 4. Chromatograph of diols derived from CVAOA.

Table 1
Substrate specificity of strain NK0505.

Substrate	Percent utilization in 4 days	Diol production	Growth (protein; µg/mL)	Substrate	Percent utilization in 4 days	Diol production	Growth (protein; µg/mL)
but-3-Enylarsonic acid	66	*rac*-3, 4-Dihydroxybutylarsonic acid	24	Ethylene	35	Ethylene glycol	21
TCE	23	Ethylene glycol	18	Isoprene	17	ND	14
PCE	0	ND	0	1,3-Butadiene	21	ND	19
				Acetylene	0	ND	0

Substrates at left were added to medium at 500 mg/mL; substrates at right were added to 30 mL headspace at 5%. ND, not determined.
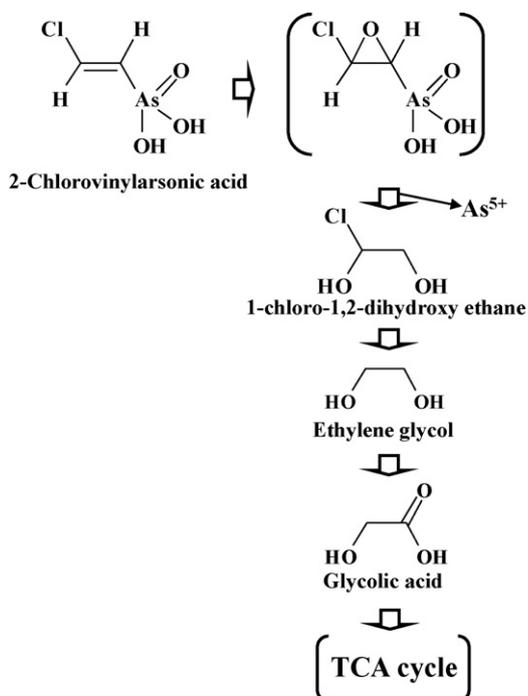
Fig. 5. Proposed degradation pathway of CVAOA. Epoxy formation by CVAOA, and the TCA cycle, have been added as references for the proposed pathway.

of CVAOA (RT 11.25) (data not shown). Further study is needed to identify this compound, which we tentatively categorized as a possible degradation product of epoxy-forming CVAOA. In the degradation of *but*-3-enylarsonic acid by strain NK0505, *rac*-3,4-dihydroxybutylarsonic acid was detected (Table 1), but in the degradation of CVAOA there were no BSTFA-derived dihydroxyl moieties containing ethylarsonic acid. This result indicated that the dihydroxylated form of CVAOA was not as stable as *rac*-3,4-dihydroxybutylarsonic acid. We also considered 1-chloro-1,2-dihydroxyethane to be a degradation product. Production of 1-chloro-1,2-dihydroxyethane by phenylboronate derivatives was observed as 4-chloro-2-phenyl-1,3,2-dioxaborolane (Fig. 4); this was confirmed by the fragmentation pattern and retention time of the synthesized 4-chloro-2-phenyl-1,3,2-dioxaborolane. Finally, 1-chloro-1,2-dihydroxy ethane was degraded to ethylene glycol (Table 1) and then glycolic acid. Glycolic acid was probably metabolized by the tricarboxylic acid cycle (Fig. 5).

4. Conclusion

We examined the microbial degradation of CVAOA. Two isolated strains, *N. carnea* NK0505 and *R. opacus* NK0506, could be grown on CVAOA as the sole source of carbon. Strain NK0505 utilized about 90% of CVAOA (50 mg/L) within 5 days; at 1000 mg/L of CVAOA no

degradation occurred over a 10-day period. We identified 1-chloro-1,2-dihydroxyethane, ethylene glycol, glycolic acid, and arsenic acid as the degradation products of CVAOA. Strain NK0505 could also degrade *but*-3-enylarsonic acid, trichloroethylene, isoprene, and 1,3-butadiene, whereas utilization of tetrachloroethylene and acetylene did not occur.

Acknowledgements

References

[1] N.B. Munro, S.S. Talmage, G.D. Griffin, L.C. Waters, A.P. Watson, J.F. King, V. Hauschild, The sources, fate, and toxicity of chemical warfare agent degradation products, Environ. Health. Perspect. 107 (1999) 933–974.

[2] K. Nakamiya, Y. Shibata, H. Ito, J.S. Edmonds, M. Morita, Synthesis of phenyl arsenic analytical standards related to contaminated well water in Kamisu, Ibaraki, Japan, Appl. Organomet. Chem. 19 (2005) 282–286.

[3] R. Haas, K. Scheibner, M. Hofrichter, Degradation of organo-arsenical chemical warfare agents by fungal enzyme manganese peroxidase, U.W.S. F. 15 (2003) 224–226.

[4] A.M. Boronin, I.T. Ermakova, V.G. Sakharovsky, G.M. Grechkina, I.I. Starovoitov, R.L. Autenrieth, J.R. Wild, Ecologically safe destruction of the detoxification products of mustard-lewisite mixtures from the Russian chemical stockpile, J. Chem. Technol. Biotechnol. 75 (2000) 82–88.

[5] R.O. Jenkins, A.W. Ritchie, J.S. Edmonds, W. Goessler, N. Molenat, D. Kuehnelt, C.F. Harrington, P.G. Sutton, Bacterial degradation of arsenobetaine via dimethylarsinoylacetate, Arch. Microbiol. 180 (2003) 142–150.

[6] C.G. Ginkel, H.G. Welten, J.A. de Bont, Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria, Appl. Environ. Microbiol. 53 (1987) 2903–2907.

[7] M.S. Shields, M.J. Reagin, Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene, Appl. Environ. Microbiol. (1992) 3977–3983.

[8] A.M. Cupples, A.M. Spormann, P.L. McCarty, Growth of a Dehalococcoides-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR, Appl. Environ. Microbiol. 69 (2003) 953–959.

[9] R. Whittenbury, K.C. Phillips, J.F. Wilkinson, Enrichment, Isolation and some properties of methane-utilizing bacteria, J. Gen. Microbiol. 61 (1970) 205–218.

[10] F.R. Siegel, Arsenic in arctic sediment cores: pathfider to chemical weapons dump sites? J. Environ. Sci. 39 (2000) 705–706.

[11] K. Ishii, A. Tamaoka, F. Otsuka, N. Iwasaki, K. Shin, A. Matsui, G. Endo, Y. Kumagai, T. Ishii, S. Shoji, T. Ogata, M. Ishizaki, M. Doi, N. Shimojo, Diphenylarsinic acid poisoning from chemical weapons in Kamisu, Japan, Ann. Neurol. 56 (2004) 741–745.

[12] K. Nakamiya, T. Nakayama, H. Ito, J.S. Edmonds, Y. Shibata, M. Morita, Degradation of arylarsenic compounds by microorganisms, FEMS Microbial. Lett. 274 (2007) 184–188.

[13] W.L. Lewis, H.W. Stiegler, The beta-chlorovinyl-arsines and their derivatives, J. Am. Chem. Soc. 47 (1925) 2546–2556.

[14] S.V. Serves, D.N. Sotiropoulos, P.V. Ioannou, E.V. Mutenda, M.J. Sparkes, H.B.F. Dixon, *rac*-3,4-Dihydroxybutylarsonic acid: a key intermediate for isosteric arsonolipids, Phosphor. Sulfur. 101 (1995) 75–82.

[15] G. Wulf, P. Birnbrich, Aldol-group-transfer polymerization of 2-phenyl-1,3,2-dioxaborole, preparation of graft and comb-like polymers, Makromol. Chem. 194 (1993) 1569–1582.

[16] G. Wulf, P. Birnbrich, A. Hansen, Synthese von oligomeren und polymeren monosacchariden durch aldol-gruppentransfer-polymerisation, Angew. Chem. 100 (1988) 1197–1198.

[17] J.A.M. Bont De, Oxidation of ethylene by soil bacteria, Anton. Leeuw. 42 (1976) 59–71.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[19] K. Nakamiya, S. Hashimoto, H. Ito, J.S. Edmonds, A. Yasuhara, M. Morita, Microbial treatment of bis (2-ethylhexyl) phthalate in polyvinyl chloride with isolated bacteria, J. Biosci. Bioeng. 99 (2005) 115–119.

[20] K. Wilson, Preparation of genomic DNA from bacteria, in: F.M. Ausbel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Short Proto-cols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, 4th ed., John Wiley & Sons Ltd., New York, NY, 1999, pp. 2-13–2-14.

[21] P. Houze, J. Chaussard, P. Harry, M. Pays, Simultaneous determination of ethy-lene glycol, propylene glycol 1,3-butylene glycol and 2,3-butylene glycol in human serum and urine by wide-bore column gas chromatography, J. Chro-matogr. 619 (1993) 251–257.

[22] D. Kanner, R. Bartha, Growth of *Nocardia rhodochrous* on acetylene gas, J. Bac-teriol. 139 (1979) 225–230.

[23] B.M. Rosner, F.A. Rainey, R.M. Kroppenstedt, B. Schink, Acetylene degradation by new isolates of aerobic bacteria and comparison of acetylene hydratase enzymes, FEMS Microbiol. Lett. 148 (1997) 175–180.

[24] J.C. Germon, R. Knowles, Metabolism of acetylene and acetaldehyde by *Rhodococcus rhodochrous*, Can. J. Microbiol. 34 (1988) 242–248.