FEMS Microbiology Letters 240 (2004) 49–53

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Microbial degradation of the organophosphate pesticide, Ethion

L. John R. Foster [a,*], Bia H. Kwan [a], Tony Vancov [b]

[a] *Biolpolymers Research Group, Centre for Advanced Macromolecular Design, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW, Australia*

[b] *New South Wales Department of Agriculture, Wollongbar Agricultural Institute, Ballina, Australia*

Received 2 May 2004; received in revised form 9 September 2004; accepted 10 September 2004

First published online 25 September 2004

Edited by C. Edwards

Abstract

The organophosphate pesticide, Ethion, remains a major environmental contaminant in rural Australia and poses a significant threat to environmental and public health. The aerobic degradation of Ethion by mesophilic bacteria isolated from contaminated soils surrounding disused cattle dip sites was investigated. Two isolates, identified as *Pseudomonas* and *Azospirillum* species, were capable of biodegrading Ethion when cultivated in minimal salts medium. The abiotic hydrolytic degradation products of Ethion such as Ethion Dioxon and *O*,*O*-diethylthiosphosphate were not detected. The data suggest the rapid degradation of Ethion to support microbial growth. The results have implications for the development of a bioremediation strategy.

Keywords: Ethion; Organophosphate; Biodegradation; *Pseudomonas*; *Azospirillum*

1. Introduction

Organophosphorus pesticides and carbamates are heavily employed in a global capacity for their broad insecticidal spectrum [1]. Ethion [(*O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′-methylene bis(phosphorodithioate))] was introduced in 1956 by the Food Machinery and Chemical Company (FMC) for use on plants and animals as an insecticide, acaricide and ovicide [2].

Ethion was used as the basis of the Australian cattle tick eradication program. Despite its cessation in the late 1970s, Ethion concentrations in dip sludges and surrounding soils remains unacceptably high, up to 45 g/kg [3]. The hydrophobic nature of Ethion plays a major role in its adsorption to soil and sediment particles reducing its runoff to natural water systems [4,5]. Similarly, studies by Sharom et al. [6] showed that Ethion is persistent in such water, undergoing little biodegradation. The environmental concentrations of Ethion and its persistence are of grave concern given that it exhibits a mammalian D_{50} of 50 mg/kg of body fat [7]. It is estimated that there are over 1600 Ethion contaminated sites in New South Wales, with similar numbers reported for other states in Australia [3].

Ethion is moderately to highly persistent in soil. Its potential for biodegradation is decreased by its hydrophobicity and strong adsorption to organic matter and soil particles [5]. As a consequence of its high toxicity and persistence, remediation of environments contaminated with Ethion is a priority. Remediation of contaminated lands in Australia has traditionally involved site management with subsequent disposal to a registered

* Corresponding author. Fax: +61 2 9313 6710.
E-mail address: J.Foster@unsw.edu.au (L.J.R. Foster).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.09.010

landfill site. There is considerable concern however for the safety of landfill disposal in relation to the long-term stability of pollutants as well as potential hazards involved in transport of contaminated waste [8]. While the hydrolysis and biodegradation of Ethion in a variety of environments has been demonstrated by Dierberger and Pfeuffer [9], to the best of our knowledge, this is the first report of its biodegradation by individual isolates which may have potential in bioremediation technologies.

2. Materials and methods

2.1. Isolation of microorganisms

Soil samples (5 g) contaminated with Ethion (22 g/kg) were used to inoculate baffled Erlen–meyer flasks containing 50 ml mineral medium supplemented with Ethion as sole carbon source [10]. Flasks were incubated at 30 °C with shaking (200 rpm) in the dark. After approximately 72 h these were then used as inocula for flasks containing the same medium which were then incubated for a further 72 h under the same conditions. Samples from these cultures were spread-plated on mineral salts agar containing 100 mg Ethion/kg. Twenty-three bacterial isolates were obtained after streaking to purity while fungal isolates were discarded. Representative isolates WAI-19 and WAI-21 were selected for further analysis.

2.2. Isolate growth in liquid culture

Isolates WAI-19 and WAI-21 were precultured in baffled Erlenmeyer flasks containing mineral salts medium with 10 mM glucose and 100 mg Ethion/l. (FMC, Sydney, Australia). Flasks were incubated at 30 °C with shaking (100 rpm) in the dark. Growth was monitored as changes in OD660 (Shimadzu, Japan). When growth had occurred, the flasks contents were centrifuged and the supernatants discarded. The cell pellets were then washed in fresh sterile medium 4 times before addition to flasks containing mineral medium and 500 mg/l Ethion. These uninoculated flasks had been preincubated for 24 h beforehand. At periodic intervals 2.5 ml samples were removed and cell growth monitored. When growth had occurred as determined by OD660 and cell viability, the flask contents were centrifuged and the supernatants discarded. The cell pellets were then washed and uniform densities produced as described above. These were then used to inoculate a series of flasks (12 per isolate), containing mineral salts medium and 500 mg/l Ethion. Flasks were incubated as described above. At periodic intervals individual flasks were sacrificed and their contents used to determine cell growth and Ethion concentration. Cultures were run in triplicate to ensure accuracy. A number of controls were performed: (1) uninoculated medium, (2) medium without Ethion inoculated with each isolate, (3) uninoculated medium with Ethion, (4) medium with Ethion inoculated with *Escherichia coli* and (5) medium without Ethion inoculated with *E. coli*.

2.3. Determination of Ethion concentration

The contents of inoculated flasks containing medium and Ethion were centrifuged and the cell pellet washed 4 times with fresh medium. The supernatants were pooled and the Ethion extracted into *n*-hexane. The solvent was then evaporated and the Ethion subsequently dissolved in acetone. The concentrations of Ethion were then determined using GC–MS (Varian, Melbourne, Australia) according to the method of Mallick et al. [11]. The efficiency of the extraction process was measured using known concentrations of Ethion in medium and determined to be 92 ± 4%.

2.4. Identification of isolates

Crude cell lysates, prepared by boiling single colonies in 50 μl of 10 mM Tris–Cl (pH 8.0) for 10 min, served as template DNA in PCR reactions. The 16S rRNA gene regions corresponding to positions 8–1543 in *E. coli* 16S rRNA were amplified using universal primer pairs, 27f and 1525r [12,13]. PCR amplification was carried out in a PE 9600 thermocycler (Perkin–Elmer, USA) using HotMaster™ *Taq* DNA polymerase under conditions specified by the supplier (Eppendorf GmbH, Germany). Amplification conditions were as follows: an initial denaturation step (94 °C for 2 min) followed by 30 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 1.5 min, and a final extension cycle of 10 min at 72 °C. PCR products were cloned into pCR2.1-TOPO by using the TOPO TA Cloning Kit (Invitrogen, Australia).

Nucleotide sequences of the cloned products were determined from plasmid DNA preparations (QiaSpin plasmid extraction kits, Qiagen GmbH, Hilden, Germany) using the ABI PRISM BigDye Terminator Cycle Sequencing kit and an ABI373 Sequencer (Applied Biosystems) according to the manufacturer's instructions. The following primers were used to sequence the genes: Universal M13F and R, 519r, 530f, 1100r, 1114f, 1392r and 1406f were used to sequence both strands of the16S rRNA genes [13].

The 16S rRNA sequences were initially screened for chimeric fragments with the CHIMERA-CHECK program version 2.7, and then compared with reference sequences contained in the GenBank Nucleotide Sequence Database using the FASTA algorithm [14–16]. The

sequence data have been submitted to the GenBank database under Accession Numbers AY563022 and AY520572, for isolates WAI-19 and WAI-21, respectively.

3. Results and discussion

3.1. Cell growth and Ethion biodegradation

Several isolates that demonstrated growth on minimal medium where Ethion was the sole carbon source were purified. Based on preliminary studies isolates WAI19 and WAI21 were selected for further study. These isolates were subsequently cultivated in liquid minimal medium in an attempt to quantify cell growth. Fig. 1 suggests that growth of strain WAI-19 could be monitored by OD660, with the growth curve attaining a maximum OD660 after 18 h. In contrast, an uninoculated control showed no change in OD660 for 20 h before gradually increasing (Fig. 1). In this case, flask sterility was confirmed through microscopic examination and plating on nutrient agar. Similarly, Dierberger and Pfeuffer have reported that the half-life of Ethion in buffered (pH 7) distilled water at 30 °C is 20.8 weeks [9]. Thus, the increase in OD660 was not due to infection or hydrolysis of the Ethion. It is most likely that agitation of the medium and hydrophobic Ethion eventually formed an emulsion increasing the OD660. Supporting this suggestion, the OD660 increase for an uninoculated control in a non-baffled flask was dramatically less (Fig. 1). Thus, the use of OD660 to monitor cell growth was deemed acceptable within a 20 h period when supported by evidence of Ethion biodegradation.

A series of 50-ml baffled flasks containing mineral salts medium and 500 mg Ethion/l were inoculated with WAI-19 and incubated at 30 °C with vigorous shaking (200 rpm). At periodic intervals three flasks were used to monitor the mean of triplicate readings for cell growth and Ethion concentration. The same procedure was carried out using WAI-21. Accurate comparison of the growth and biodegradation patterns between the two isolates was facilitated by using similar viable cell inoculation densities. Figs. 2(a) and (b) clearly show that cell growth during the first 20 h was coupled with the degradation of Ethion during the same period.

During the first 6–7 h incubation WAI-19 and WAI-21 exhibited an initial rapid degradation of Ethion of approximately 30 and 42 mg Ethion/l/h, respectively. This was subsequently followed by a slower rate of approximately 3–4 mg Ethion/l/h, eventually attaining 58% and 70% degradation for WAI-19 and WAI-21 respectively (Fig. 2). An uninoculated control and a control of medium inoculated with *E. coli* alone showed no change in Ethion concentration (Fig. 2). Cessation of biodegradation activity may have been due to complete consumption of an essential limiting nutrient, which may also play a role in its environmental biodegradation. Clearly, an effective bioremediation technology based on such isolates requires further research investigating the factors affecting their biodegradation of Ethion.

The biodegradation of Ethion by WAI19 and WAI21 (t_{50}s: 11.5 and 9.5 h, respectively) is somewhat faster

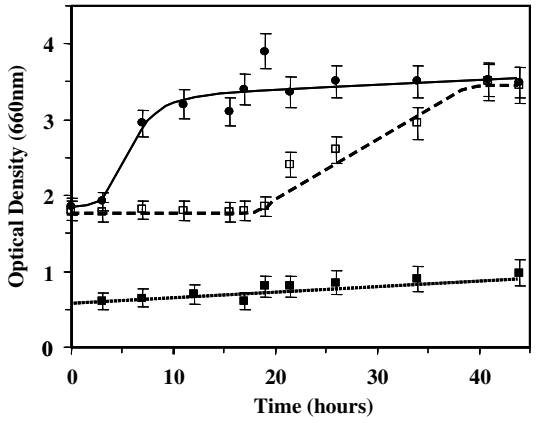

Fig. 1. Cell growth as monitored by optical density for isolate WAI-19 when incubated at 30 °C in mineral salts medium (pH 7, 200 rpm) with Ethion as sole carbon source, (●) WAI-19, (□) uninoculated control and (■) uninoculated control with reduced agitation.

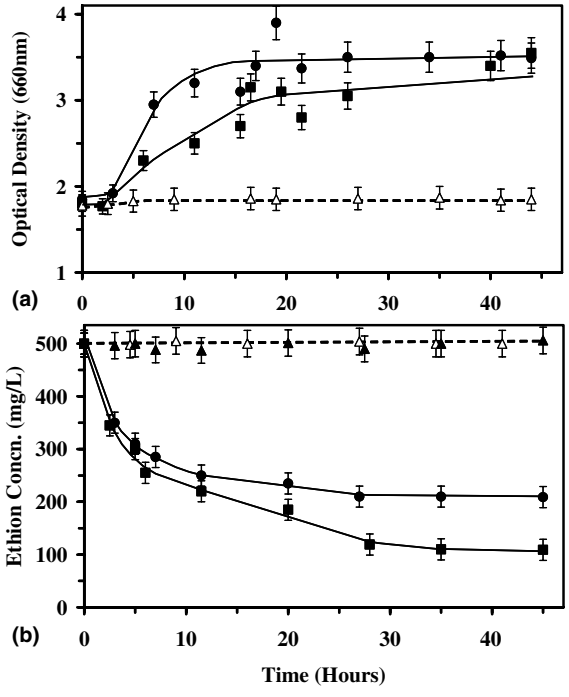

Fig. 2. Cell growth and corresponding degradation of Ethion as sole carbon source for isolates WAI-19 and WAI-21 at 30 °C, (pH 7, 200 rpm). (a) Growth of isolates WAI-19 (●) and WAI-21 (■), inoculated with *E. coli* control (△). (b) Degradation of Ethion by WAI-19 (●), WAI-21(■), *E. coli* (▲) and an uninoculated control (△).

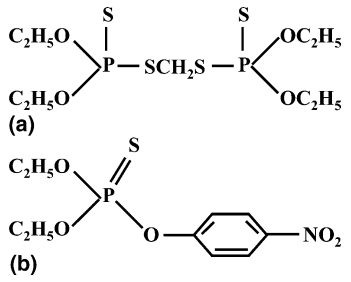

Fig. 3. Chemical structure of Ethion (a) and Parathion (b).

than that reported for the organophosphate Methylparathion by a *Pseudomonas* sp. (t_{50}: 17 h) [17]. Ethion differs structurally from Parathion and other organophosphorus insecticides such as Coumaphos and Diazinon, in its lack of an aromatic or heterocyclic group (Fig. 3). Sethunathan and Yoshida demonstrated that a *Flavobacterium* sp. was able to hydrolyse Parathion and Diazinon with the liberation of the heterocyclic and aromatic moieties [18]. The lack of these components in Ethion may support its significantly faster microbial degradation. The abiotic hydrolytic degradation products of Ethion; Ethion Monoxon, Ethion Dioxon, *O*,*O*-diethylthiophosphate and Thio-formaldehyde were not observed using GC–MS [9,19]. Their absence may be due to a number of factors, however it seems most likely that Ethion and its degradation products were rapidly utilised for microbial growth.

3.2. Identification of isolates

The sequence of approximately 1474 bp of the 16S rRNA gene of WAI-19 was 98.8% identical to that of the 16S rRNA gene of *Azospirillum* sp. DA2-3-1 (GenBank Accession No. AY118224), 98.0% with *Azospirillum* sp. Mat2-1a (AY118222), 97.0% with *Azospirillum* sp. Arm2-2 (AF521650), and 97.3% similar to that of the 16S rRNA gene of *Azospirillum lipoferum* strain *ncimb11861* (AL16SRRN5). Based on the high degree of sequence similarity, WAI-19 was putatively identified as a member of the genus *Azospirillum*. Isolate WAI-21 was recognized as a member of the genus *Pseudomonas*, with similarities of 99.8% with the 16S rRNA sequence of *Pseudomonas* sp. P400Y-1 (GenBank Accession No. AB076857) [20], 99.7% with *Pseudomonas* sp. YG-1 (AF441203) [20], 99.6% with *Pseudomonas* sp. (PSP-IPA1) [21], and 97.6% with *Pseudomonas citronellolis* (Z76659). While *A. lipoferum* is though to play a role in the biodegradation of plant matter and is known to biodegrade aromatics [22,23], *P. citronellolis* is reported to biodegrade polyhydroxyalkanoates (PHAs) [20]. It is not known whether either of these species can biodegrade organophosphorous compounds.

The high environmental concentrations of Ethion found in the vicinity of cattle and sheep dip sites pose a significant health risk. Despite the apparent persistence of Ethion in these environments, our study shows that it can serve as a source of carbon supporting microbial growth. Two isolates, WAI-19 and WAI-21, identified as *Azospirillum* and *Pseudomonas* species, respectively, rapidly biodegraded Ethion. The application of such species in bioremediation technologies is currently under investigation.

References

[1] Fytianos, K., Konstantinou, J., Kaidatzis, J. and Albanis, T. (1998) Multiresidue determination of insecticides and fungicides in fruits and vegetables by gas chromatography. Bull. Environ. Contam. Toxicol. 61, 684–689.
[2] United States Environmental Protection Agency. (1993) Manual of Chemical Methods for Pesticides and Devices. AOAC International, Washington, DC, USA.
[3] Barzi, F., Naidu, R. and McLaughlin, M.J. (1996) Contaminants and the Australian soil environment In: Contaminants and the Soil Environment in the Australia-pacific Region (Naisu, R., Kookana, R.S., Oliver, D.P., Rogers, S. and McLaughlin, M.J., Eds.), pp. 451–484. Kluwer Academic Publishers, London, UK.
[4] Sharom, M.S., Miles, J.R.W., Harris, C.R. and McEwen, F.L. (1980) Behaviour of 12 insecticides in soil and aqueous suspensions of soil and sediment. Water Res. 14 (8), 1095–1100.
[5] Howard, P.H. (1991) Handbook of Environmental Fate and Exposure Data for Organic Chemicals–Pesticides, Vol. 3. Lewis Publishers, Chelsea, MI, USA, pp. 5–13.
[6] Sharom, M.S., Miles, J.R.W., Harris, C.R. and McEwen, F.L. (1980) Persistence of 12 insecticides in water. Water Res. 14 (8), 1089–1093.
[7] Food Machinery and Chemistry Plc Ltd. (2001) Safety Data Sheet for Ethion.
[8] Burns, R.G., Rogers, S. and McGhee, I. (1996) Remediation of inorganics and organics in industrial and urban contaminated soils In: Contaminants and the Soil Environment in the Australia-pacific Region (Naisu, R., Kookana, R.S., Oliver, D.P., Rogers, S. and McLaughlin, M.J., Eds.), pp. 411–449. Kluwer Academic Publishers, London, UK.
[9] Dierberger, F.E. and Pfeuffer, R.J. (1983) Fate of Ethion in canals draining a Florida citrus grove. J. Agri. Food Chem. 31 (4), 704–709.
[10] Shelton, D.R. and Somich, C.J. (1988) Isolation and characterisation of coumaphos metabolising bacteria from cattle dip. Appl. Environ. Microbiol. 54 (10), 2566–2571.
[11] Mallick, K., Bharati, K., Banerji, A., Shakil, N.A. and Sethunathan, N. (1999) Bacterial degradation of chlorpyrifos in pure cultures and in soil. Bull. Environ. Contamin. Toxicol. 62, 48–54.
[12] Brosius, J., Palmer, J.L., Kennedy, J.P. and Noller, H.F. (1978) Complete nucleotide sequence of a 16S rRNA gene from Escherichia coli. Proc. Natl. Acad. Sci. USA 75, 4801–4805.
[13] Lane, D.J. (1991) 16S/23S rRNA sequencing In: Nucleic Acid Techniques and Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. Willey Publishers, Chichester, UK.
[14] Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. and Tiedje, J.M. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31 (1), 442–443.
[15] Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A. and Wheeler, D.L. (2000). GenBank Nucleic Acids Res. 28 (1), 15–18.

[16] Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence analysis. Proc. Nat. Acad. Sci. USA 85, 2444–2448.
[17] Ramanthan, M.P. and Lalithakumari, D. (1999) Complete mineralisation of methylparation by *Pseudomonas* sp. A3. Appl. Biochem. Biotechnol. 80 (1), 1–12.
[18] Sethunathan, N. and Yoshida, T. (1973) A *Flavobacterium* that degrades diazinon and parathion. Can. J. Microbiol. 19, 873–875.
[19] Ivey, M.C. and Mann, H.D. (1975) Gas–liquid chromatographic determination of Ethion, Ethion monoxon and Ethion dioxon in tissues of turkeys and cattle. J. Agri. Food Chem. 23 (2), 319–321.
[20] Khan, S.T., Horiba, Y., Yamamoto, M. and Hiraishi, A. (2002) Members of the family comamonadaceae as primary Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. Appl. Environ. Microbiol. 68 (7), 3206–3214.
[21] Wilson, A.E., Moore, E.R. and Mohn, W.W. (1996) Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. Appl. Environ. Microbiol. 62 (9), 3146–3151.
[22] Widmer, F., Shaffer, B.T., Porteous, L.A. and Seidler, R.J. (1999) Analysis of nifH gene pool complexity in soil and litter at a Douglas Fir forest site in the Oregon Cascade mountain range. Appl. Environ. Microbiol. 65 (2), 374–380.
[23] Lopez-de-Victoria, G. and Lovell, C.R. (1993) Chemotaxis of *Azospirillum* species to aromatic compounds. Appl. Environ. Microbiol. 59 (9), 2951–2955.