Biodegradation **8:** 357–361, 1998.

Short Communication

Biodegradation of alkylpyridines by bacteria isolated from a polluted subsurface

Zeev Ronen*, Aharon Abeliovich & Ali Nejidat
Environmental Microbiology Unit, Ben-Gurion University of the Negev, The J. Blaustein Institute for Desert Research, Sede Boker Campus, 84990, Israel, Fax: 972-7-659-6831; (author for correspondence)*

Accepted 2 January 1998

Key words: alkylpyridine, subsurface bacteria, biodegradation

Abstract

Ten bacterial strains were isolated from alkylpyridine polluted sediments 7.6 m below the surface. These strains were able to degrade 11 different alkylpyridine isomers. Degradation rates depended on number and position of the alkyl group. Isomers with an alkyl group at position 3 were more resistant to microbial attack. Of the 10 strains, 6 isolates were selected for detailed study. These isolates mineralized the isomers to CO_2, NH_4^+, and biomass. All strains were gram-negative rods with a strict aerobic metabolism. Characterization of physiological and biochemical properties revealed similarity between strains. Eeach strain however, had a limited substrate range which enabled it to degrade no more than 2 to 3 compounds of the 14 alkylpyridine isomers tested. Examination of the genetic variability among cultures with the randomly amplified polymorphic DNA technique revealed high levels of genomic DNA polymorphism. The highest similarity between 2 strains (0.653) was observed between 2-picoline and 3-picoline degrading cultures. The molecular basis of the differences in substrate specificity is under investigation.

Introduction

Contamination of groundwater by pyridine and pyridine derivatives has been documented at numerous sites (Sims and O'Loughlin 1989, Ronen and Bollag 1995). The release of pyridine and its derivatives into the environment is the result of processes that take place during the production of fuel from coal and oil-shale, the extraction of creosote from coal-tar, the utilization of creosote in the wood-preservation industry and the extraction of chemicals from coal-tar (Ronen and Bollag 1995). Pyridines can migrate quickly through the soil as observed by Leenheer and Stuber (1981) and any inappropriate disposal of these compounds on the soil surface will eventually result in rapid leaching of the pollutants into the subsurface and groundwater.

Degradation of alkylpyridines by soil microorganisms has been reported (Kost et al. 1978, Shukla 1984, Sims and O'Loughlin 1989, and Ronen and Bollag 1995). In addition to surface soil microorganisms, the potential of subsurface microorganisms to degrade alkylpyridines was demonstrated under aerobic and anaerobic conditions (Rogers et al. 1985, Kaiser and Bollag 1992, Ronen et al. 1996). However, axenic cultures that degrade these chemicals were not isolated from the subsurface. The objective of this study therfore, was to isolate and characterize alkylpyridine-degrading bacteria from a contaminated subsurface soil.

Materials and methods

Isolation of pure cultures

Enrichment cultures for alkylpyridine-degrading bacteria were initiated by inoculating 100ml of mineral salt medium (Kaiser and Bollag 1992) with 5 g of contaminated sediment 7.6 m below the soil surface (Ronen

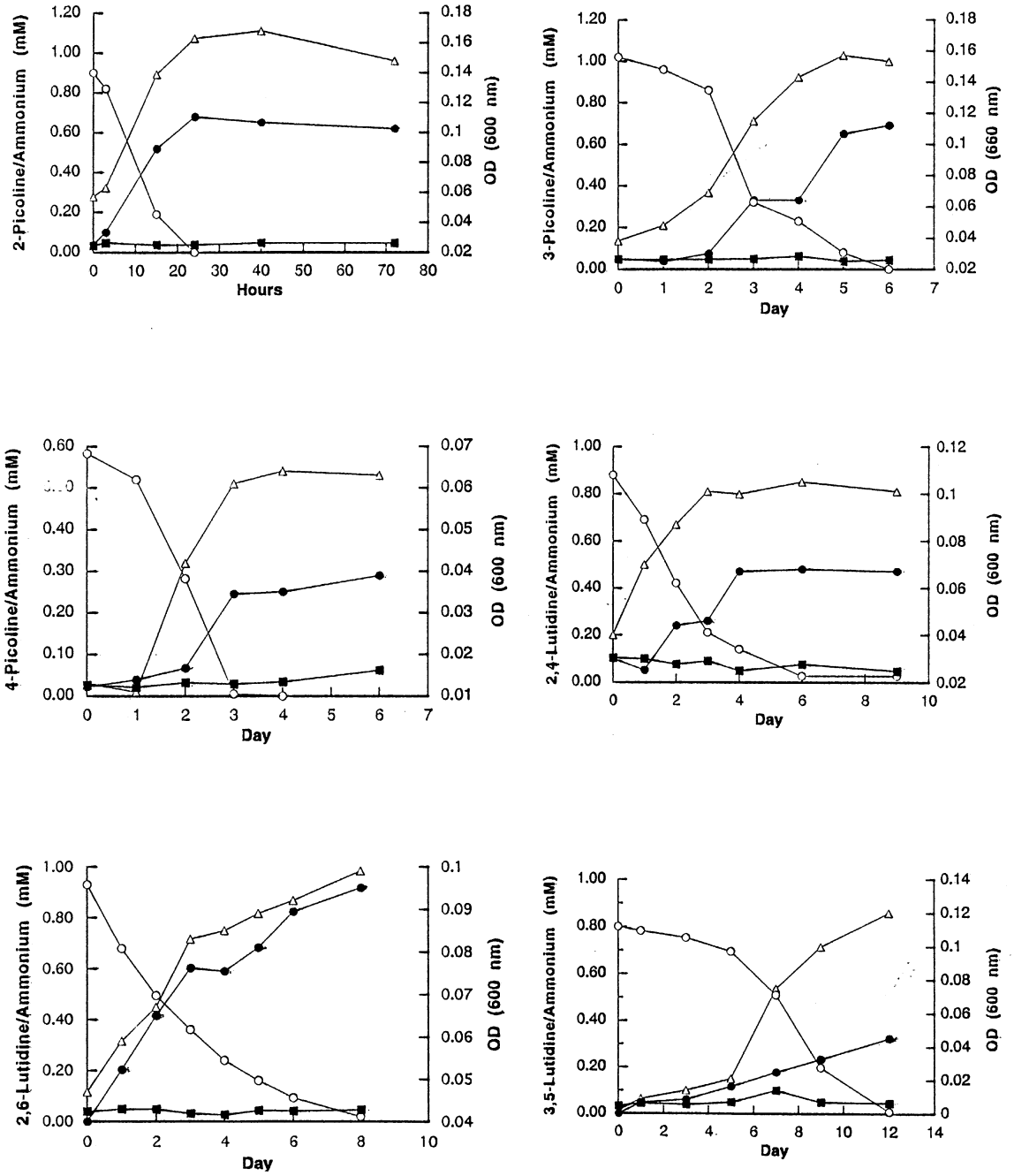

Figure 1. Biodegradation of 2-,3-, and 4-Picoline and 2,4-, 2,6-, and 3,5-Lutidine by pure cultures isolated from polluted subsurface.

et al. 1996). Enrichment was achieved by successive transfers of cultures into fresh medium containing the appropriate alkylpyridine isomer every 2 weeks. These cultures were used to inoculate agar plates containing mineral medium and a single alkylpyridine (200 mg/l). Single colonies were selected from the plates and further purified by repetitive streaking on the agar plates. Cultures were maintained on agar slants with the appropriate substrate and stored at 4 °C. Isolated strains were examined for morphological and biochemical proper-

Table 1. Mineralization of alkylpyridines by pure cultures to CO_2 and NH_4^+. Cultures were incubated for 8 days with the respective alkylpyridine isomer. Residual ammonium, DOC and the substrate were measured at the end of the incubation

Isolate	Dissolved organic carbon (mg/l)			Alkylpyridine (mM)	NH_4^+ (mM)
	Day 0	Day 8	% Mineralized	Day 0	Day 8
2-Picoline	105.2	6.9	93.5	1.13	0.61
3-Picoline	77.0	6.2	92.0	0.83	0.50
4-Picoline	77.8	3.9	95.0	0.84	0.33
2,4-Lutidine	88.9	18.8	77.6	0.78	0.52
2,6-Lutidine	78.4	12.6	84.0	0.73	0.39
3,5-Lutidine	79.9	8.7	89.1	0.74	0.33

ties by the API 20NE kit (BioMérieux, Lyon France). All alkylpyridine isomers were obtained from Aldrich Chemicals (Milwaukee, Wisconsin).

Analytical methods

High performance liquid chromatography, HPLC, was used for analysis of the various compounds as previously reported (Ronen et al. 1996). Ammonium in the culture medium was analyzed with Nessler reagent (Clesceri et al. 1989). Dissolved organic carbon (DOC) was determined using a Dohrmann DC-190 TOC analyzer (Rosemount Analytical Inc., Santa Clara, CA). Analysis of randomly amplified polymorphic DNA (RAPD) was performed as previously described (Khandka et al. 1996). Similarity between strains was estimated as described by Wang and Tanksley (1989).

Results and discussion

Isolation and characterization of pure cultures

Thirteen different enrichment cultures were established from the subsurface contaminated sediment and ten strains of alkylpyridine-degrading bacteria were isolated. Attempts to isolate pure cultures that degrade 2,3-lutidine, 2,5-lutidine, 3,4-lutidine and 2-methyl-5-ethylpyridine were unsuccessful. All the tested isolates were able to use the respective alkylpyridine as the sole carbon and nitrogen sources (Figure 1). This study is the first to describe the isolation of pure alkylpyridine degrading cultures from the subsurface. Our inability to isolate pure cultures that degrade 2,3-lutidine and 3,4-lutidine may suggest that the degradation of these compounds requires the activity of more than one organism. This assumption is supported by the observation that a stable mixed culture cultivated in the chemostat was able to metabolize these compounds (data not shown). The nature of the interaction among microorganisms in the mixed culture that facilitates the biodegradation of these compounds was not determined.

All isolates were gram-negative rods and had a strict aerobic metabolism (data not shown). Although some of the strains had denitrification capacity, nitrate could not be used as an alternative electron acceptor for the biodegradation of alkylpyridines under anaerobic conditions (i.e. 2-picoline, 4-picoline, and 2,6-lutidine degraders). Except for the 2,4-lutidine degrader which was identified as *Pseudomonas (Burkholderia) capacia* , none of the other strains was identified by the use of the API 20NE kit.

Mineralization of alkylpyridine by pure cultures

Pure cultures were incubated with different alkylpyridines for 8 days. Residual compound, dissolved organic carbon, and ammonium were determined (Table 1). In all of the cultures the respective substrate was not detected by HPLC at the end of the incubation period. The extent of carbon and nitrogen mineralization varied between 77.6% and 95% for carbon and 39.2% and 66.6% for nitrogen. The ability of these bacteria to mineralize the chemicals makes them a good tool for the bioremediation of polluted soil (Table 1). Release of ammonium in alkylpyridine contaminated soil as a result of microbial activity has been previously observed (Sims and Sommers 1985). Thus, it is now clear that mineralization of the compound can also occur in the polluted subsurface soil.

Table 2. Substrate specificity of different pure cultures of bacteria isolated from the polluted subsurface sediment. Each isolate was incubated separately with 14 different alkylpyridine isomers as sole carbon and nitrogen source. Positive marks were given to cultures that in addition to growth on the tested compound released ammonium into the culture medium

Compound	2-Picoline degrader	3-Picoline degrader	4-Picoline degrader	2,4-Lutidine degrader	2,6-Lutidine degrader	3,5-Lutidine degrader
Pyridine	–	–	++	++	–	+++
2-Picoline	+++	++	++	–	–	+++
3-Picoline	–	+++	–	–	–	–
4-Picoline	–	–	+++	++	–	+++
2-Ethylpyridine	+++	++	++	–	–	Not determined
3-Ethylpyridine	–	+++	–	–	–	–
4-Ethylpyridine	–	–	++	++	–	–
2,3-Lutidine	–	–	–	–	–	–
2,4-Lutidine	–	–	++	+++	–	–
2,5-Lutidine	–	–	++	++	–	–
2,6-Lutidine	–	–	–	–	+++	–
3,4-Lutidine	–	–	–	–	–	–
3,5-Lutidine	–	–	–	–	–	+++
2,4,6-Collidine	–	–	–	–	+++	Not determined

Substrate specificity and genetic similarity of the different strains

Some similarity in substrate specificity was observed between the 2-picoline, 3-picoline and 3,5-lutidine-degrading strains, and between the 4-picoline and 2,4-lutidine-degrading strains (Table 2). Moreover, pure cultures isolated on 2, 3, and 4-ethylpyridine had a substrate range identical to 2, 3, and 4-picoline-degrading isolates. In addition, the 2,4,6-collidine-degrading strain had the same substrate range as the 2,6-lutidine degraders and, thus, were considered as the same isolates. Substrate specificity in biodegradation of alkylpyridines by soil bacteria has been reported (Shukla 1975). In this study the substrate range of the different isolates was thoroughly examined (Table 2). In contrast to the biodegradation of pyridine derivatives, reports on the degradation of homocyclic compounds indicated that pure cultures are able to degrade benzene and most of its alkyl isomers (Haligler et al. 1992; Oh et al. 1994). The structural differences between the pyridine and benzene ring may explain these phenomena. While the benzene ring is symmetric, the nitrogen atom of the pyridine ring breaks this symmetry. For example, while there is only one monomethyl benzene isomer (toluene) there are three monomethyl pyridine isomers (2-, 3-, and 4-picoline). Thus, it is likely that a specific enzyme(s) is used for the initial degradation step of each alkylpyridine.

The ability to degrade alkylpyridine was lost after culturing the isolates on non-selective medium. However, our attempts to isolate plasmids using various methods, failed to detect any in all six isolates. This may indicate that the genes coding for enzymes involved in alkylpyridine degradation are located in the genome. Analysis of 164 amplified DNA fragments obtained by 6 random 10-mer primers revealed low genomic DNA similarity between the genomes of the six isolates described. Similarity coefficients were 0.24 to 0.653 The only significant similarity (0.653) obtained was between the isolates that degrade 2- and 3-picoline. The low genomic DNA homology as indicated by the RAPD analysis may imply that the different strains have not evolved from one common ancestor. These findings are in line with the narrow substrate specificity observed.

In conclusion, our findings demonstrate that subsurface microorganisms are capable of degrading a class of heterocyclic pollutants and suggest possible biotreatment of alkylpyridines polluted sites. The results show that the different isolates have a unique degradation capacity and therefore, any treatment of a polluted site should involve the use of a mixed culture rather than of a single bacterial strain.

Acknowledgments

Isolation of alkylpyridine degrading bacteria was carried out in the Laboratory of Soil Biochemistry at The Pennsylvania State University (USA) under the supervision of Dr. J.-M. Bollag. This study was funded in part by a post doctoral fellowship to Z.R. from The Blaustein International Center and a Deichman Fellowship to A.N. We would like to thank Simon Barak for editing the manuscript.

References

Clesceri LS, Greenberg AE & Trussell RR (Eds) (1989) Standard methods for the estimation of water and wastewater (pp 4–117). Port City Press, Baltimore, Maryland

Haligler BE, Pettigrew CA & Spain JC (1992) Biodegradation of mixture of substituted benzene by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. 58: 2237–2244

Kaiser JP & Bollag JM (1992) Influence of soil inoculum and redox potential on the degradation of several pyridine derivatives. Soil Biol. Biochem. 24: 351–357

Khandka DK, Nejidat A & Golan-Goldhirsh A (1996) Polymorphism and DNA markers for asparagus cultivar identified by random amplified polymorphic DNA. Euphytica 87: 39–44

Kost AN, Vorob'eva LI, Terent'ev PB, Modyanova LV, Shibilkina OK & Korosteleva LA (1978) Microbiological transformation of pyridine derivatives. Appl. Biochem. Microbiol. 13: 541–546

Leenheer JA & Stuber HA (1981) Migration through soil of organic solutes in an oil-shale process water. Environ. Sci. Technol. 15: 1467–1475

Oh YS, Shareefdeen Z, Baltzis BC & Bartha R (1994) Interactions between benzene, toluene, and *p*-xylene (BTX) during their biodegradation. Biotech. Bioengin. 44: 533–538

Rogers JE, Riley RG, Li SW, O'Malley ML & Thomas BL (1985) Microbial transformation of alkylpyridines in groundwater. Water Air Soil Pollut. 24: 443–454

Ronen Z & Bollag JM (1991) Pyridine metabolism by a denitrifying bacterium. Can. J. Microbiol. 37: 725–729

— (1995) Biodegradation of pyridine and pyridine derivatives by soil and subsurface microorganisms. Intern. J. Environ. Anal. Chem. 59: 133–143

Ronen Z, Bollag JM, Hsu CH & Young JC (1996) Feasibility of bioremediation of a groundwater polluted with alkylpyridines. Groundwater 34: 194–199

Shukla OP (1975) Microbial decomposition of 2-ethylpyridine, 2,4-lutidine, and 2,4,6-collidine. Ind. J. Exp. Biol. 13: 574–575

— (1984) Microbial transformation of pyridine derivatives. J. Scient. Ind. Res. 43: 61–122

Sims GK & Sommers LE (1985) Degradation of pyridine derivatives in soil. J. Environ. Qual. 14: 580–584

Sims GK & O'Loughlin EJ (1989) Degradation of pyridines in the environment. CRC Critic. Rev. Environ. Cont. 19: 309–340

Wang ZY & Tanksley SD (1989) Restriction fragment length polymorphism in *Oryza sativa* L. Genome 32: 1113–1118.