Bull. Environ. Contam. Toxicol. (1992) 49:10–17

Bulletin of Environmental Contamination and Toxicology

Screening Test of the Biodegradative Capability of a New Strain of *Pseudomonas gladioli* (BSU 45124) on Some Xenobiotic Organics

Timothy D. Dawson and Fu-Hsian Chang

Center for Environmental Studies, Bemidji State University, Bemidji, Minnesota 56601, USA

The extent of environmental pollution throughout the world has resulted in a need for new innovative technologies to clean up waste streams at the site of generation and to clean up previously contaminated sites. Although biological treatment of wastewaters and sewage has been used since the activated sludge process was first developed in 1914, we have only recently recognized the value of biological treatment of contaminated soils, groundwater, and novel industrial wastes (Garg 1989). Bioremediation in conjunction with physical and chemical processes has been effectively used in the field to decontaminate solvent contaminated groundwater, and pesticide contaminated soil, surface water, and groundwater (Bourquin 1989). It has also been successfully demonstrated for cleanup of diesel fuel and waste oil (Mathewsen and Grubbs 1989).

The Pseudomonads are taxonomically heterogeneous and nutritionally very versatile (De Vos 1981). Because of this great versatility they are of great scientific and practical importance to man. They have been found to be readily adaptable to various man-made compounds such as the chlorinated aromatic hydrocarbons (Kobayashi and Rittman 1982). The species that have been the most thoroughly studied are *Pseudomonas cepacia*, *P. putida*, and *P. aeruginosa* (Vecht et al. 1988; Spain et al. 1989; Spain and Gibson 1988; Harwood et al. 1990; Folsom et al. 1990; Nelson et al. 1988).

This series of experiments was designed as a screening test to determine the potential of the microorganism, *Pseudomonas gladioli* BSU 45124, to biodegrade organic compounds of environmental concern. Leisinger et. al. (1981) refer to the initial enzymatic transformation of the parent compound as primary biodegradation. Primary biodegradation in these experiments means the structure of the parent compound is enzymatically changed enough that it can no longer be detected by either UV spectrophotometry or gas chromatography. Most of these experiments used the test compound as the sole source of

Send reprint requests to Dr. Fu-Hsian Chang at the above address.

carbon in the growth medium. This was an attempt to identify if the organism was using a mineralization process or not. In some instances phenol was used as an additional carbon source to induce the production of enzymes that might have a broad enough substrate specificity to result in primary biodegradation of the test compound. The compounds chosen covered a wide spectrum of degradability from solvents to slightly recalcitrant compounds, such as the mono-chlorophenols, to compounds known to be extremely persistent such as trichlorophenols.

MATERIALS AND METHODS

The bacterium was isolated in our lab by Gu and Chang (1989). The organism, a gram negative rod, is indigenous to the reed-sedge peat bogs of northern Minnesota. A sample of the organism was sent to the American Type Culture Collection for identification. They identified the organism as a strain of *Pseudomonas gladioli*, according to Bergey's manual (Palleroni 1984). They based their identification on a comparison with the type strain and noted that it was atypical in a few characteristics (ATCC 1989). We have designated this strain BSU 45124. This strain differed from the Bergey's description and the type strain by showing growth at 4°C and utilization of the following compounds as sole carbon sources: Mesaconate, L-Rhamnose, lactose, and phenol.

The chemicals have been grouped into three classes for ease of discussion. Cell growth and cultivation differed between classes so they will be discussed separately, beginning with the experiment testing for biodegradative capabilities on o-, m-, p-cresols, phenol, o-xylene, toluene, and benzene (Class 1 chemicals), and 2-, 3-, 4-chlorophenol, 2,4-dichlorophenol, and 2,3,4-trichlorophenol (Class 2 chemicals).

For the Class 1 and 2 chemicals, cells were initially grown aerobically at 25°C on a rotary shaker. The medium used was peptone (0.5%), glycerol (1.0%), yeast extract (0.1%) in distilled water, adjusted to a pH of 7.0-7.5. After 36-48 hours cells were harvested by centrifuging for 20 minutes at 10,000 rpm. The supernatant was discarded and cells were centrifuged and rinsed 3 times with a 0.05*M* (pH 7.5) phosphate buffer solution. Cells were resuspended in a phosphate-buffered mineral salts medium, PAS (Bedard et al. 1986). Twenty mL of this cell solution were placed in 30 mL, amber colored, screw cap vials with teflon lined septa. Toluene and benzene experiments were incubated in 30 mL, amber colored, crimp sealed, vials with teflon lined septa.

An alternate procedure was used for growth of cells on phenol prior to biodegradation tests. Two loops of bacteria, grown on polypeptone, glycerol, and yeast extract (PGY) agar slants, were inoculated in 100 mL of PAS medium. The flasks were shaken and then phenol from a 10,000 µg/mL stock solution (in distilled water) was added to give a final concentration of 200 µg/mL in the flask. After 24, 36, and 38 hours,

additional phenol was added to increase the concentration by 150-200 µg/mL. After 48 hours 100 µg/mL phenol was added and one hour later the medium was centrifuged and the cells washed and resuspended using the same procedure as for the PGY grown cells.

All tests were set up with 3 replicates and 3 controls that were not inoculated with cells. Tests were begun with the addition of stock solutions of chemicals to various starting concentrations. The concentrations tested were not toxic to this microorganism yet still at environmentally significant levels. Flasks were then incubated at ambient temperature on a rotary shaker at 125 rpm.

Cell growth and cultivation for experiments using 2,4-dichlorophenoxyacetic acid (2,4-D), pentachlorophenol (PCP), and phenoxyacetic acid (PAA) (Class 3), were as follows. Cells were grown aerobically at 25°C on a rotary shaker. The medium was a phosphate-buffered mineral salts mixture, PAS (Bedard et al. 1986). It contained phenol and 2,4-D as carbon sources and was supplemented with 0.005% yeast extract.

Phenol and 2,4-D were added to 100ml flasks of medium and the final concentrations were 200 and 50 ug/ml respectively. Cells were inoculated into this supplemented PAS medium from agar slants previously described. Flasks were incubated aerobically on a rotary shaker. After 24 and 36 hours, additional phenol was added at the same concentrations as initially in medium. After 48 hours the cells were harvested by centrifuging. Cells were resuspended in PA concentrate to an optical density of 0.5 at 540 nm. Seventy-five mLs of this cell solution were placed in 300 mL shake flasks with screw caps.

All tests were set up with 3 replicates and dead cell controls. Control cells were inactivated by the addition of mercuric chloride to a final concentration of 1m*M* (Bedard et al. 1986). Tests were begun with the addition of stock solutions of 2,4-D, PCP, PAA, and phenol, in separate flasks, at initial concentrations of 50, 50, 75, and 200 ug/mL respectively. 2,4-D and PCP flasks were also started with 200 ug/mL phenol as an enzyme inducer.

The class 1 and class 2 chemicals were analyzed by removing aliquots at time zero and various times afterward. The aliquots were centrifuged and/or extracted and the concentrations of the test compounds were determined by gas chromatography. UV spectrophotometric determinations were used to detect disappearance of the class 3 compounds using a Beckman Model 34 Spectrophotometer with 1 cm quartz cuvettes. 2,4-D, PCP, and PAA were measured at their respective lambda max of 283, 318, and 268 nm (Loos et al. 1967; Trevors 1982). Phenol was measured at 283 nm to be sure it would not interfere with 2,4-D absorbance readings. Absorbance readings were compared to standard curves of each compound in an unamended PAS medium. UV scans were performed in all tests to check for changes in shape of

absorbance peaks that were not detected at the lambda max. Control flasks were run through same procedures as sample flasks and UV scans were run to check for any non-biological effect on absorbance of tested compounds.

At time zero and various times afterward, 6-7 mL aliquots were removed from each flask. Samples were then centrifuged at 4,000 rpm for 45 minutes to separate the cellular material. Five milliliters of the resulting supernatant was transferred to a test tube, acidified to pH 2 with 1N H_2SO_4, and extracted with 5 mL ethyl ether (Rosenburg and Alexander 1980). Tubes were placed on a Tekmar VXR shaker at 1400 rpm for 15 minutes, and let stand for 5 minutes afterward to allow phases to separate before reading with UV spectrophotometer.

The non-chlorinated aromatic hydrocarbons were analyzed on a Perkin Elmer Sigma 3B gas chromatograph equipped with a flame-ionization detector. The column used was a 0.1% Alltech AT-1000 on Graphpac GC (80/100, 6' x 1/4", I.D. 2 mm). The carrier gas was nitrogen at a flow rate of 27 mL/minute, injector and detector temperatures were 225° C and 250° C respectively. The oven was run under isothermal conditions of 80° C (benzene), 160° C (toluene), and 210° C (cresols). The extent of degradation was determined by external standard method. Comparisons of peak areas to standard curves were used to determine concentrations of test compounds.

The chlorinated compounds of class 2 were analyzed on a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector (^{63} Ni). A fused silica capillary column , DB 608 (15M x 0.53 mm, 0.83 µm FSOT, proprietary phase), from J & W Scientific (Folsom, CA) was used for each analysis. The carrier gas was helium at 4.5 mL/minute with nitrogen as a make-up gas at 40 mL/minute. The injector and detector temperatures were 225° C for the mono-chlorophenol isomers and 250° C for the di-, and tri- chlorophenols. The oven was held at isothermal conditions of 115° C (2-chlorophenol), 120° C (3-, 4-chlorophenol, 2,4-dichlorophenol), and 140° C (2,3,4-trichlorophenol). One microliter injections of pentane extracts were made in a splitless injector with 0.5 minute purge time. Quantitation was carried out using a Hewlett Packard 3392A integrator, and the extent of degradation was determined by the external standard method.

Experimental design required the removal of aliquots from the same sample vial for time zero and each subsequent measurement. Therefore the aliquots are not independent. Time also needed to be included as an independent variable as well as the presence or absence of viable cells. This experimental design allows the use of two-way mixed design factorial analysis of variance for determination of significant factors (Jaccard 1983). This statistical method showed the interaction between the two independent variables (time, cells) and the one dependent variable (chemical concentration). It determined if there was an interaction effect. Significance level (P) of a variable is based on the

observed P value. The variable is considered significant when $P < .05$.

RESULTS AND DISCUSSION

Pseudomonas gladioli BSU 45124 is shown to have a broad enzyme substrate specificity for the primary biodegradation of aromatic hydrocarbons. As shown in Table 1, the bacterium was able to utilize phenol, benzene, toluene o-cresol, m-cresol, p-cresol, 2-chlorophenol, and 4-chlorophenol as sources of carbon. Table 1 lists the percent degradation, corrected for any loss in control vials due to non-biological degradation, along with the starting concentrations of the chemicals.

The data for the degradation of phenol was analyzed using Students t-test. The results of this test were $t(3) = 95.09$, $p < 0.01$. Growth of the bacteria on phenol, prior to inoculation, induced the production of the enzymes responsible for degradation of toluene. This resulted in no detectable levels of toluene after 24 hours of incubation. When cells were grown on PGY medium, with similar initial concentrations prior to inoculation, significant levels of toluene remained after 96 hours of incubation. The inducibility of an enzyme is potentially important, if it has a broad enough specificity to initiate the degradation of more recalcitrant compounds. The following compounds showed no statistically significant degradation by PGY grown cells after the incubation periods indicated: Toluene (96 hr), 3-chlorophenol (72 hr), 24-dichlorophenol (25 days), 2,3,4-Trichlorophenol (25 days), 2,4-Dichlorophenoxy acetic acid (28 days), and pentachlorophenol (19 days).

Table 1. Percent degradation after incubation period for compounds that showed statistically significant degradation by *Pseudomonas gladioli* (BSU 45124).

Compound	Incubation Period	Initial Concentration (µg/mL)*	Percent Degradation**
Phenol	48 hr.	200.0	98.4
Benzene	24 hr.	53.7	81.6
Toluene (Phenol grown)	22 hr.	44.1	40.1
o-Cresol	48 hr.	106.6	100.0
m-Cresol	48 hr.	103.0	99.3
p-Cresol	40 hr.	105.9	97.7
2-Chlorophenol	24 hr.	8.78	74.6
4-Chlorophenol	24 hr.	2.96	100.0

* Means of 3 replicates in sample vials at time zero.
** Adjusted for losses in 3 replicates of control vials.

Presence of one chlorine in the ortho- or para- positions of the phenol molecule did not block the degradative capabilities of this microorganism as seen in Table 1. The inability to significantly degrade 3-chlorophenol is consistent with the results of other researchers who reported that increased resistance to biodegradation was due to meta substitution of chlorine (Alexander and Aleem 1961).

Multiple substitutions of chlorine, as in the di- and trichlorophenols tested, did make the phenol molecule resistant to degradation. The use of phenol or one of the monochlorophenols as an inducer for the degradation of the more highly chlorinated compounds was not tried, but will be the focus of our future research.

The preliminary examination of this microorganism is significant in that it is the first evidence we have found, reporting on the capabilities of Pseudomonas gladioli to degrade various aromatic hydrocarbons including the monochlorinated phenols. The species has been known primarily as a plant pathogen to Gladiolus species, onions (Allium), and birds rest fern (Asplenium ridus) (Mortenson et al. 1988; Kishun and Swarup 1982; Chase et al. 1984). Some strains have also been shown to be producers of unidentified antibiotic substances and an antifungal substance (Wakimoto et al. 1986; Mao and Capellini 1989). *Pseudomonos gladioli* has also recently been isolated from the respiratory tract of Cystic Fibrosis patients but there is no apparent association with infectious complications of the disease (Mortenson et al. 1988; Christenson et al. 1989). A literature review failed to produce any evidence of studies done on the biodegradative abilities of this species.

The identification of microorganisms with an ability to degrade common environmental contaminants is an important step in increasing our understanding of pollution problems and possible solutions. Elucidation of the metabolic pathways involved in this degradation may allow us to manipulate or maximize them for our use in waste treatment and bioremediation of contaminated sites.

The extensive use of toluene and benzene in industry and the presence of these compounds as well as the cresols in various waste streams and contaminated environmental sites leads us to believe that this organism should be investigated further for possible use in bioremediation. In addition, its potential for degrading chlorinated compounds should be further investigated. Although laboratory investigations using single substrates in pure cultures can't be extrapolated to the more complex natural environment, they are the starting point for identifying important organisms and enzymatic pathways. More screening tests are being conducted to evaluate the biodegradative capability of a broader scope of xenobiotic organics by this bacterial strain.

Acknowledgments. Funding for this project was supplied by the Center for Environmental Studies, Bemidji State University, Bemidji, MN.

REFERENCES

Alexander M, Aleem MIH (1961) Effect of chemical structure on microbial decomposition of aromatic herbicides. J Agr Food Chem 9:44-47

American Type Culture Collection (1989) Project report SC 2045, bacterial identification. ATCC, Rockville, MD

Amy PS, Schulke JW, Frazier LM , Seidler RJ (1985) Characterization of

aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl Environ Microbiol 49:1237-1245

Bedard DL, Unterman R, Bopp LH, Brennan MJ, Haberl ML, Johnson C (1986) Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl Environ Microbiol 51:761-768

Bourquin AW (1989) Bioremediation of hazardous waste. Hazardous Materials Control Sept.-Oct. 2:16-59

De Vos P (1981) Intra and intergeneric similarities of ribosomal ribonucleic acid cistrons in and with the genus *Pseudomonas*. Mededelingen Van De Koninklijke Academie Voor Wetenschappen, Letter, en Schone Kunsten Van Belgie, Klasse Der Wetenschappen. 43:23-60

Folsom BR, Chapman, PJ, Pritchard PH (1990) Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates. Appl. Environ. Microbiol. 56:1279-1285

Garg S (1989) Biotechnology, microbes, and pollution control: Potentials and barriers. ASTM Standardization News 17:44-49

Gu Z-Y, Chang F-H (1989) Biodegradation of toxic organic fraction in wet carbonized peat filtrates. In: Spigarelli SA (ed) Proc. Int. Symposium on Peat/Peatland Characteristics and Uses. May 16-20, 1989. Bemidji State University, Bemidji, MN pp. 536-551

Harwood, CS, Parales, RE, Dispensa, M (1990) Chemotaxis of *Pseudomonas putida* toward chlorinated benzoates. Appl. Environ. Microbiol. 56:1501-1503

Jaccard J (1983) Statistics for the behavioral sciences. Wadsworth Publishing Company, Belmont, CA pp 342-384

Kobayashi H, Rittman, BE (1982) Microbial removal of hazardous organic compounds. Environ. Sci. Technol. 16: 170A-183A.

Leisinger T, Hutter R, Cook AM, Nuesch J, (eds) (1981) Microbial degradation of xenobiotic and recalcitrant compounds. FEMS Symposium No. 12, Academic Press, London, p 43

Loos MA, Roberts RN, Alexander M(1967) Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species. Can J Microbiol 13:679-690

Mathewsen JR, Grubbs RB (1989) Commercial microorganisms. Hazmat World June:48-51

Nelson MJ, Montgomery SO, Pritchard PH (1988) Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. 54: 604-606.

Palleroni NJ (1984) Genus 1. *Pseudomonas* Migula 1894. In: Krieg NR, Holt JG (eds) Bergey's Manual of Systematic Bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 141-143

Rosenburg A, Alexander M (1980) 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) decomposition in tropical soils and its cometabolism by bacteria in vitro. J Agric Food Chem 28:705-709

Spain JC, Zylstra GJ, Blake CK, Gibson, DT (1989) Monohydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in

Pseudomonas putida F1. Appl. Environ. Microbiol. 55: 2648-2652.
Spain JC, Gibson DT (1988). Oxidation of substituted phenols by *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS6. Appl Environ Microbiol 54: 1399-1404
Vecht SE, Platt MW, Er-El Z, Goldberg, I (1988) The growth of *Pseudomonas putida* on m-toluic acid and on toluene in batch and in chemostat cultures. Appl Microbiol Biotechnol 27: 587-592
Trevors JT (1982) Effect of temperature on the degradation of pentachlorophenol by *Pseudomonas* species. Chemosphere 11:471-475
Ware GW (1989) The pesticide book. Thomson Publications, CA. pp 3-16

Received September 15, 1991; accepted January 19, 1992.