PRELIMINARY INVESTIGATION OF ORGANISMS CAPABLE OF DEGRADING DIELDRIN AND AROMATIC COMPOUNDS: TECHNIQUES FOR ISOLATION AND CHARACTERIZATION

Brad Harten

INTRODUCTION

The degradation of most organic compounds is usually due to microbial activity since nonenzymatic reactions seldom lead to the breakdown of introduced chemicals. Many microorganisms have been shown to degrade aromatic and other hydrocarbon compounds.

Two different catabolic processes are responsible for the removal of chemicals from the environment. The first, primary metabolism, refers to the mineralization process in which carbon and energy are derived from the breakdown of the organic molecule by the microorganism. The second, cometabolism or cooxidation, refers to the process in which biotransformation of the chemical occurs without the net production of energy or carbon. Organisms use carbon and energy derived from a second substrate for growth and to carry out the cometabolic transformation.

The distinction between these two processes may have significant ramifications. Mineralization results in the removal of organic, and potentially toxic, compounds from the environment. Because these compounds are used as substrates, they may be eliminated relatively quickly. Cometabolic processes, however, generally do not transform the chemical quickly since compounds subject to attack are not used as substrates for energy. In addition, only small changes in molecular structure may result from these transformations. Cometabolic products similar to the original compound may then accumulate in the environment. Byproducts may even be more toxic than the parent compound. Horvath (1972) stated that cometabolism may be important in the degradation of recalcitrant molecules including halogenated pesticides such as DDT and aldrin.

Interestingly, the enzymes involved in some aromatic degradative pathways have been shown to be plasmid-encoded, particularly in Pseudomonas species (Williams and Worsey, 1976). This is usually the primary metabolic enzymes. The role of plasmids in cometabolism is unclear.

The purpose of this study was to test for the presence of primary and cometabolic organisms in the marine environment and to develop techniques for their isolation. Both aromatic compounds and the insecticide dieldrin were used as test compounds. Partial characterization of organisms known to metabolize aromatics was carried out using plasmid screening procedures and thin layer chromatography. One isolate which produced clearing zones on dieldrin was also screen for plasmids.

MATERIALS AND METHODS

All minimal salts broth and plates were prepared using the procedure of Baumann and Baumann, Chapter 104, p. 1308. The Prokaryotes. Neopeptone broth

dieldrin solution (50mg/20ml). Plates of both neopeptone and minimal salts media were used in the original replica plating technique. Agar overlayer plates were made by adding 0.4 ml of the 50 mg/20ml stock solution to 5ml of minimal salts agar medium. The 5 ml of overlayer agar was added to prepoured agar plates of either minimal salts or neopeptone agar.

Agar digesting isolates were grown in liquid culture using 0.1% agar in either neopeptone or minimal salts broth.

Soil samples were collected as 1cm by 2cm plugs from two plots of extra high fertilized plots in the Great Sippewissett Marsh. The plugs were pooled and one gram samples were placed in liquid enrichment culture. Liquid enrichment cultures contained 50ml of minimal salts media with aromatics and dieldrin added at 1mg/ml. After three days samples were taken and serial dilutions performed. These dilutions (10^{-2}, 10^{-3}, 10^{-4}) were plated using 0.1 ml/plate on minimal salts agar and neopeptone agar plates for spread plates and only minimal salts plates using agar overlayer technique. Samples obtained from J. Romesser were also tested using the agar overlayer technique and grown on minimal salts media in plates saturated with aromatic vapors. These plates contained filter papers containing the aromatics from stock solutions. Aromatic metabolizers were scored for growth under aromatic vapors. Marsh isolates on agar overlayers were checked for clearing zones around suspected aromatic utilizers. Since under visible light no clearing zones were detected, long wavelength UV light was used. The aromatics used fluoresence at these wavelengths.

Plasmid Isolation Procedure

The procedure of Kada and Liu (1981) and of Birnboim and Doly (1979) were used for plasmid isolation.

Thin-Layer Chromatography

Thin-layer chromatography was used to identify metabolites from possible cometabolizing yeast strains. Yeast cultures were grown in 25 ml neopeptone broth. pH was reduced to below 5. Cells were centrifuged and the supernatant decanted into a 50ml centrifuge tube. A solvent/solvent extraction was performed using equal volumes of ethyl acetate. Tubes were shaken for 3-5 min for each extraction. The upper phase was decanted after each extraction and placed into a 125 ml flask. Extractions were repeated 3 times. Anhydrous Na_2SO_4 was added in a thin layer to remove any water.

Metabolites were concentrated using flash evaporation in a round-bottomed flask. When most of the solvent was evaporated, acetone was added in three 1 ml aliquots to dissolve any remaining metabolites. Acetone solutions were placed in a small screw cap vials. The acetone was then evaporated under an N_2 gas jet leaving a thin film in the bottom of the vial. Before using, 0.2 ml acetone was added to each vial to resuspend metabolites.

Ten microliters of unknowns and standard solutions were spotted onto Kodak 13181 silica gel thin layer chromatogram sheets and placed into a 10:1 toluene:acetic acid solvent for 45 min. Chromatograms were sprayed with Gibbs reagent and heated 10 min at 100°C.

RESULTS

Screening for aromatic and dieldrin metabolizers

The replica plating technique of Kiyohara, et al. (1982) showed no clearing zones and was abandoned. The agar overlayer technique using visualization of clearing zones under UV light produced good results. Several colonies which produced clearing zones on dieldrin, naphthalene, phenanthrene, and biphenyl were isolated. Most isolates proved to be agar degraders. One agar degrading isolate was shown to be a gram positive coccus. Colonies were either yellow or tan. Colonies isolates from naphthalene plate tended to actinomycete-like organisms. Those isolated from phenanthrene and biphenyl plates tended to be agar degraders.

Organisms sent by J. Romesser, except 7078, all grew well on biphenyl. Phenethrene and naphthalene both tended to inhibit growth of these organisms (Table 1).

Plasmid Isolation

Biphenyl degraders from J. Romesser were screened for plasmids. The gram positive coccus isolated for dieldrin plates was also screened. Both screeing procedures showed the presence of plasmids in the agar degrader. None of the biphenyl metabolizers produced plasmids. Plasmids of known size were used as standards. The plasmid found appeared to be approximately 35 mg daltons in size.

Thin-Layer Chromatography

A wide range of metabolites was found among the four yeast strains tested. However, few corresponded to the standard solutions of compounds known to occur during cometabolism of biphenyl in yeasts. Compounds with R_f values of approximately .14, .21 and .49 occured in all four unknown strains, but did not correspond to standards. Metabolites which appear to have similar R_f values include in unknown 3510:4-phenylphenol; unknown 3530: O-phenylphenol and 4-phenylphenol.

The results obtained to date are tentative. The main purpose of this study was to develop a procedure for the detection of microorganisms which can utilize aromatic and other recalcitrant compounds. The procedures outlined above allow for the detection of these organisms for the marine environment.

RESULTS OF BIPHENYL METABOLISM IN CULTURES FROM J. ROMESSER

Organism	Biphenyl	Naphthalene	Phenanthrene	Salts
7077	++	+	(+)	-
7078	(+)	(+)	(+)	(+)
7079	++	-	-	-
7080	++	(+)	(+)	+
3020	++	+	(+)	++
3021	++	-	(+)	+
3022	++	-	-	++
3023	++	(+)	(+)	-
3024	++	(+)	(+)	++
3025	++	(+)	(+)	-
3026	++	(+)	(+)	(+)
3027	++	(+)	+	(+)

++ : heavy growth
+ : moderate growth
(+): slight growth
- : no growth

THE EFFECT OF NEUROTOXINS ON CHEMOTAXIS IN *Spirochaeta aurantia*

P.L. Hartzell

Spirochaeta aurantia is a spirochaetal organism distinguished by its unique morphology and motility. The helical shaped cell has a protoplasmic cylinder encased in axial fibrils which has been compared to flagella in other bacteria. The number of axial fibrils varies between different spirochaetes, but in all spirochaetes, the axial fibril is embedded at one end of the protoplasmic cylinder and the other end is not. These fibrils have been shown to play a role in motility in spirochaetes although the mechanism is unknown. Metabolic inhibitors such as sodium arsenate or carbonyl cyanide-M-chlorophenyl hydrazone have been used to establish the relationship between motility and proton motive force. Proton motive force, specifically the membrane potential has been shown to be involved in the chemotactic response in *Spirochaeta aurantia*. Experiments using the ionophore valinomycin have further demonstrated that membrane potential fluctuations may provide chemotactic sensory signals. Although much of the chemotactic response sequence is known, the exact signal between receptors and motility organelles has evaded researchers. In their normal energized state, cells will swim and tumble (or flex) at random, when a chemoattractant such as D-xylose is detected, the cells become excited and will swim up a concentration gradient. They will remain in this excited state for a brief period before returning to the adapted stated. This adapted state is induced by the methylation of specific proteins, the methyl-accepting chemotactic proteins. L-methionine has been shown to be necessary for chemotaxis but not for motility as S-adenosyl methionine is needed for the methylation of these proteins.

Several hypotheses have been suggested to explain the mechanism of signal transduction in chemotaxis. A number of studies have demonstrated that changes in the membrane potential are involved in the chemotactic response. The intracellular calcium concentration may also be involved in chemotaxis as an increase in the level of calcium causes increased tumbling in *E. coli*. A third explanation may relate to the cyclic GMP levels. An increase in the cyclic GMP concentration has been shown to stimulate the methylation of methyl accepting chemotactic proteins. It has also been reported that levels of cGMP increase in the cell in response to chemoattractants.

The experiments I carried out this summer were based on work initiated by Eric Goulbourne who showed that certain neurotoxins and a calcium ionophore inhibited the chemotactic response to D-xylose in capillary assays. These neurotoxins used were known to affect action potential generation and transmission in excitable eucaryotic cells. The inhibitors examined in my studies included Botulinum toxin A, aconitine, tetrodotoxin, tetraethylammonium chloride and calimycin. Botulinum toxin is known to interfere with calcium movement in cells. Aconitine is a lipid soluble toxin which inhibits the closing of sodium channels and allows other cations to pass through once an action potential has been generated. It causes prolonged depolarization and blocks the return to the resting state. Tetrodotoxin, a potent neurotoxin from puffer fish binds to sodium channels in stimulated cells. Tetrodotoxin is a heterocyclic guanidine which is positively charged at neutral pH. Chemically tritiated tetrodotoxin has been used very successfully in binding studies to sodium channels. Tetraethylammonium chloride (TEA), a chemical which inhibits conductance and the calcium ionophore A23187 (calimycin) were also tested.

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The purpose of these experiments was to ascertain whether or not these inhibitors were affecting chemotaxis by inhibiting growth or whether there was a block in the excitation or adaptation response to the chemoattractant.

MATERIALS AND METHODS

Bacterial strain and culture conditions:

Spirochaeta aurantia M1 was obtained from Dr. P. Greenberg. The organism was cultured aerobically at 30 C in TGY medium containing 0.2% D-glucose; 0.5% trypticase (BBL); and 0.2% DIFCO yeast extract. The pH was adjusted to 7.5 and autoclaved. A 2% inoculum was used and stocks were transferred daily. Cultures used for photomicroscopy work were 38-42 hrs old and had an OD at 660nm of 0.10 to 0.12.

Basal medium for swarm plate migration assays contained 0.2% trypticase; 0.1% DIFCO yeast ext., 0.5% DIFCO agar and 0.02% D-glucose. The pH was adjusted to 7.5 with 4M KOH prior to autoclaving. The medium was cooled to 55 C and 1% (vol/vol) sterile 1M KPi pH 7 was added. Ten ml of medium was used for small petri plates and 19ml for large plates.

Chemicals

Tetrodotoxin, aconitine, botulinum, TEA and calimycin were obtained from SIGMA. Stock solutions of TEA tetrodotoxin, botulinum and calimycin were prepared in distilled H_2O and filter sterilized through a 0.23μ filter. Aconitine was dissolved in 100% methanol and was not treated further. Sterile stock solutions used throughout these experiments were: botulinum 10μg/ml; TEA 1M; calimycin 19mM; aconitine 7.75mM and tetrodotoxin 3mM.

Growth assays

Concentrations of neurotoxins and calimycin found to inhibit chemotaxis in capillary assays were used in TGY broth to determine the effects of these chemicals on the growth of S. aurantia. All tests were done in triplicate and each tube was examined periodically for contamination. The final concentrations in 10ml of TGY medium were: tetrodotoxin 3μM; aconitine 100μM; botulinum 100ng/ml; TEA 5mM and calimycin 100nM. The pH of the medium was unaffected by addition of any of these compounds. A 32 hr culture of S. aurantia OD 0.11 at 660nm was used to inoculate each tube (4% inoculum). One ml samples were removed at 8-12 hr intervals for optical density readings at 660nm. A Gilford model 2600 spectrophotometer with a 0.3mm slit width and 2cm pathlength was used.

Swarm Plate Migration Assays

TGY medium with 0.5% agar was used to measure migration rates of S. aurantia in the presence of neurotoxin or ionophore. The inhibitors were added to give the final concentration found to inhibit chemotaxis in the capillary assays. Plates were spotted in the center with 4μl of a 28-32 hr culture of S. aurantia. The diameter of the growth was measured every 12 hrs for 4-6 days. The rate of migrtion for each time period was taken as the average of 5-10 plates. Migration rings were also examined for sharp vs fuzzy bands relative to plates lacking any inhibitor.

Darkfield microscopy photographic assay

A Zeiss inverted microscope equipped with a darkfield condensor and a Hg lamp was employed to observe the response of S. aurantia cells to the chemoattractant D-xylose in the presence and absence of the various neurotoxins or calimycin. For these studies, 40-44 hr cultures of S. aurantia grown in TGY broth were centrifuged at 15-20 C 16,000xG for 15 min. Pellets were resuspended 1:2 in chemotaxis buffer containing 5mM glucose, 200µM cysteine-HCl and 10mMKPi pH7. Aliquots of this suspension were mixed with inhibitors or buffer such that the final dilution of cells was 1:4. Final concentrations of neurotoxins or calimycin were identical to those used in other assays. 30µl of each suspension was placed on a well polished cover slip and examined under the inverted darkfield microscope. Several photographs were taken, each at 2-4 sec exposure times. Kodak plus X black and white film (ASA 125) was used. At T=0 sec, 5µL of 125mM D-xylose was added to the drop with a Hamilton syringe and carefully mixed. Additional photographs were then taken at 25-45 sec intervals for 5 min after addition of the chemoattractant.

RESULTS

Growth assays:

Addition of any of the inhibitors except tetraethylammonium chloride at the concentrations found to inhibit chemotaxis did not inhibit growth. Fig. 1 shows growth curves for S. aurantia with and without inhibitor present. In the presence of 5mM TEA, there was a 24 to 30 hr lag prior to onset of growth. Incubation was continued for an additional 5-6 days but growth in TEA continued to lag and after 8 days was only 50-55% of the controls.

Swarm plate migrations:

The response to chemoattractant in swarm plates with and without inhibitor was tested. In two of the three tests, aconitine, TEA and tetrodotoxin inhibited the average rate of migration and increased the initial lag period. However, the appearance of the bands of growth did not appear to be significantly different. Fuzzy rings were noted with aconitine and tetrodotoxin during the first 24 hrs, but growth appeared to be normal after that time. The results of the migration assays are shown in Table 1 and graphically in Fig. II. It is important to note that although the overall average migration rate and the final diameter are lower, the rates are almost identical after the first 60 hrs. This may be due to some adaptation of the part of the organism or to breakdown of the neurotoxin over time in the agar medium. The latter idea is supported by the fact that delay of 24 hrs or more in initial spotting of these plates resulted in a shorter lag period and reduced the inhibition. It would be useful to test this on large (150 x 15mm) plates with successive spottings.

Darkfield microscopic assays

The microscopic assays were, at best, difficult to interpret. Each photograph was counted for total number of cells, swimming and flexing. Each picture contained 40-60 cells. Although the data are reported as percentage of cells swimming, some other differences were noted which are more difficult to

ascertain in the 2-4 sec exposures. With the neurotoxin botulinum, the excitation-adaptation response quantitatively appeared to be normal. However, the flexing cells appears to round up more frequently and would appear as swirls in the photographs. The duration of each swim also seemed to increase in some of the neurotoxins. The length of the run appeared longer as compared to the same time exposure for cells in buffer. The results of each sequence of photographs are shown in Fig. III-VI. Although the exact numbers and times are different for each test, the pattern is determined from three separate experiments. Tetrodotoxin appeared to inhibit the initial response to addition of chemoattractant. In all three tests, the number of flexing cells increased upon addition of D-xylose. In addition, the number of swimming cells was never as high as the same batch of cells in buffer. In the presence of 100 μM aconitine, the spirochaetes had greatly reduced or no response to addition of D-xylose. When cells were resuspended in 200μM aconitine, there was no response at all and 95-100% of the cells were flexing. In controls with methanol alone, the cells appeared to respond normally to the chemoattractant. Tetraethylammonium chloride also seemed to affect the excitation response. Upon addition of D-xylose, there was a lag of 30-40 sec duration prior to swimming response. Botulinum and calimycin did not alter the proportions of swimming and flexing cells in response to chemoattractant.

DISCUSSION

These results implicate sodium channel involvement in the chemotactic response. Motility alone and growth are unaffected by the neurotoxins aconitine and tetrodotoxin whereas chemotaxis is impaired. These are both compounds which are known to affect sodium channels. Tetraethylammonium chloride was also found to alter the spirochaetes reaction to a chemoattractant. This would support the hypothesis that membrane potential fluctuations are involved in the signal response. These three inhibitors all blocked the reaction at the level of initial excitation while the adaptation eventually appeared to be unaffected. If aconitine is blocking the closing of sodium channels as it does in eucaryotic cells, this would indicate that intracellular sodium levels must decrease in response to a chemoattractant as part of the signal. The lack of inhibition by botulinum and calimycin indicate that calcium concentrations in the cell may not be as significant as earlier reported.

TABLE I MIGRATION RATES OF S.aurantia ON SWARM PLATES

TIME(HRS)	0	24	42	64	90	132	RATE(AVE) mm/hr	%
CONTROLS(12)*	6**	9.2	14.3	20.9	27.8	41.4mm	0.268	100
BOTULINUM(3)	6	8	12.8	18.5	24.5	33	0.20	75
TEA (6)	6	11	20	28	36.3	45.7	0.30	112
CALIMYCIN(5)	6	10.5	14.6	21.2	27.2	39.5	0.25	93
TETRODOTOXN(5)	6	7.9	8.7	11.7	16.5	24.5	0.14	52
ACONITINE (6)	6	7.5	8.8	14	19.1	27.3	0.16	59

* NUMBERS IN () INDICATE NUMBER OF PLATES AVERAGED.
** NUMBERS INDICATE AVERAGE DIAMETERS IN mm
CONCENTRATIONS: TETRODOTOXIN,3uM; TEA 1mM; ACONITINE,100uM; BOTULINUM 100ng/ml; CALIMYCIN 100nM.

TIME(HRS)	0	17	41	77	99	137	% OF CONTROL
CONTROL	5	8.3	19.5	34	42	59	100
TEA 1mM	5	7.5	8	8	12	25	42
TEA 5mM	5	7.7	18	33	39	54	91
TEA 10mM	NO GROWTH						

PLATES WERE SPOTTED WITH 3ul OF A 36hr. CULTURE OF S.aurantia O.D 1.1 WITHIN 5 HRS OF POURING PLATES.

TIME(HRS)	0	23.5	42	64	90	132
CONTROLS	6	9.3	14.3	21	28	42
RATE*	0.14	0.27	0.3	0.27	0.33	
BOTULINUM	6	8	13	19	24	33
RATE	0.08	0.27	0.27	0.20	0.21	
TETRODOTOXIN	6	8	8.7	11.7	16.5	24.5
RATE	0.08	0.04	0.13	0.19	0.19	
ACONITINE	6	7.5	8.8	14	19	27.5
RATE	0.06	0.07	0.23	0.19	0.20	
CALIMYCIN	6	10.5	14.6	21.2	27.5	39.5
RATE	0.19	0.22	0.29	0.24	0.29	

* RATES ARE GIVEN FOR EACH PERIOD RATHER THAN THE OVERALL AVERAGE