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School of Civil and Environmental Engineering

Aerobic Biodegradation of Diphenylamine

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Aerobic Biodegradation of Diphenylamine

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ABSTRACT

Diphenylamine (DPA) is a primary pollutant widely used as a stabilizer in explosives and as precursor for pesticides, pharmaceutical products and dyes. It has recently been found that a dioxygenase enzyme is responsible for the ability of bacteria to biodegrade diphenylamine (8). Despite our understanding of the degradation pathway of DPA, important questions remain regarding the evolutionary origin of the gene that encodes the dioxygenase. The answers to these questions will help future efforts to biodegrade this hazardous pollutant. Through selective enrichment, isolation and genetic screening, aniline degrading bacteria have been discovered to be responsible for the genes that encode the lower part of the DPA degradation pathway. Using the same technique of enrichment, isolation and genetic sequencing, we screened for candidates for the origin of the genes that encode DPA dioxygenase. Our hypothesis is that carbazole dioxygenase is the progenitor of DPA dioxygenase at the Repauno site. Comparison of the dioxygenase genes will allow us to test the hypothesis that horizontal gene transfer facilitated assembly of the pathway. We have also hypothesized that DPA degrading bacteria will not be able to degrade carbazole as a sole carbon, nitrogen and energy source to determine whether or not degrading bacteria are capable of having two pathways of degradation. We will test DPA degraders to determine whether or not they are able to degrade carbazole. Analyzing carbazole degradation will allow us to test the hypothesis that DPA degrading bacteria will not have retained the ability to degrade carbazole during the evolution of DPA dioxygenase due to our hypothesis that DPA dioxygenase originated from carbazole dioxygenase. In our future studies we are hoping that a carbazole degrader will encode a carbazole dioxygenase that will show significant similarities to DPA dioxygenase. We hope to develop results what will suggest that

the dioxygenase gene originated from carbazole degraders through horizontal gene transfer. Experiments revealed that carbazole was not degraded by DPA degraders. The data support our hypothesis that DPA degrading bacteria did not retain the carbazole degradation pathway from carbazole degrading bacteria during the evolution of DPA dioxygenase.

INTRODUCTION

I. Diphenylamine Dioxygenase

Diphenylamine (DPA) is widely used as a stabilizer in explosives and propellants and as a precursor for pesticides, pharmaceuticals products and dyes (2). DPA is listed as a primary pollutant; it is indicated along with its derivatives as potentially hazardous to aquatic organisms (2,3). Certain bacteria can degrade DPA because they have a particular enzyme, known as diphenylamine dioxygenase that catalyzes the initial step in DPA degradation.

The purpose of this project is to provide a better understanding and knowledge of the evolution of the DPA biodegradation pathway that has been observed at contaminated sites. Both the sequence similarity and the gene organization suggest that the DPA degradation pathway was derived from the two gene clusters that encode the initial dioxygenase gene and the aniline degradation pathway (8). The bacteria that degrade aniline clearly provided the genes for the downstream pathway whereas the source of the DPA dioxygenase is still in question. My hypothesis is the DPA dioxygenase evolved from carbazole dioxygenase at the Repauno site originated from carbazole and aniline degrading bacteria. We are also hypothesizing that DPA degraders will not be able to degrade carbazole as the sole carbon, nitrogen and energy source. Here we propose to not only test the hypothesis that the DPA degradation pathway recently evolved by gene recruitment from carbazole and aniline degrading bacteria but also test the ability of DPA degrading isolates to degrade carbazole as a carbon, nitrogen and energy source. The results of this project will increase our understanding of the pathway evolution for biodegradation of synthetic chemicals. Knowledge of the origin of the DPA dioxygenase gene

will allow further research into the biodegradation of this primary pollutant at manufacturing sites.

II. Previous work on evolutionary implication and origin of diphenylamine Dioxygenase (done)

Until recently, research on biodegradability of DPA and its derivatives were sparse, and the mechanisms and the organisms that are responsible for the biodegradation of DPA were unknown (1). Biodegradation pathways that have been reported to be similar to the biodegradation of DPA are carbazole, dibenzo-p-dioxin, dibenzofuran, and diphenylether (5,6,7,2). Previous work in our laboratory led to isolation of the aerobic bacteria, *Burkholderia sp*. Strain JS667 and *Ralstonia sp*. Strain JS668 from a DPA contaminated site. These were able to use DPA as a carbon, nitrogen and energy source. The DPA degradation pathway and the genes involved were determined in the environmental isolates (8).

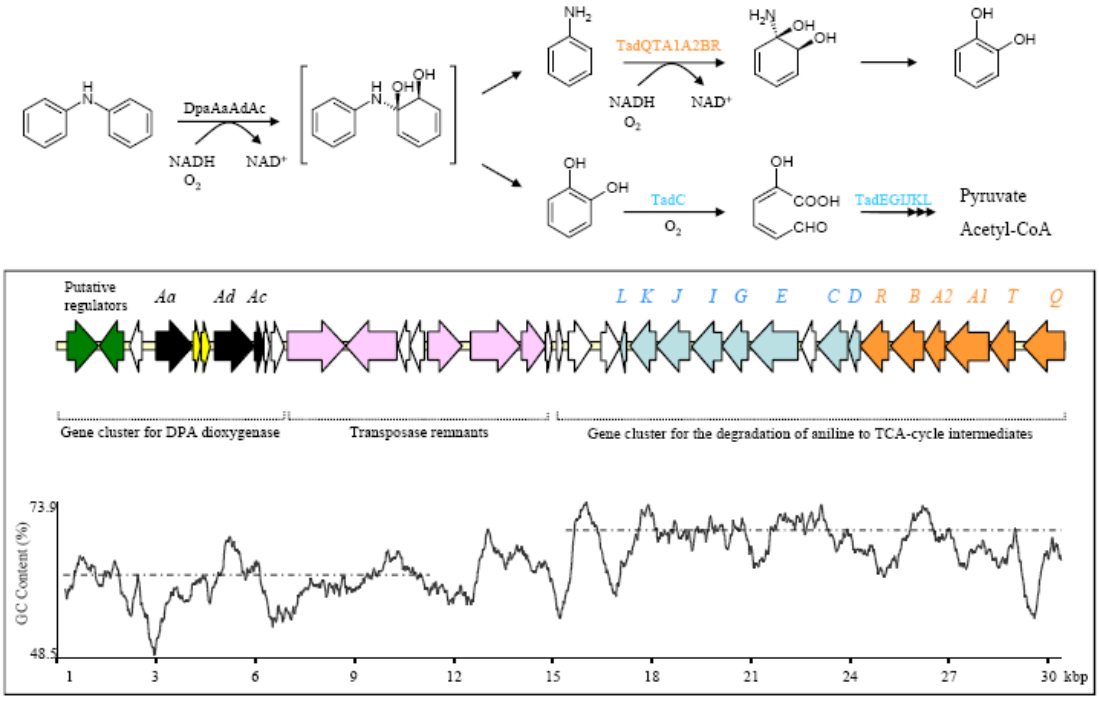

Figure 1. Biodegradation pathway of DPA and the genes involved in the degradation (top) Biodegradation pathway of DPA (bottom) genes involved in the degradation and GC content of the gene cluster (30,579 bp) encoding the complete degradation of DPA from JS667 (Adapted and modified from reference 8 with permission)

III. Carbazole as secondary candidate

Both the sequence similarity and the gene organization suggest that the DPA degradation pathway was derived from the two gene clusters that encode the initial dioxygenase gene and the aniline degradation pathway (8). Genetic exchange between these two types of bacteria may be responsible for the evolution of DPA degradation at the contaminated sites (8). With solidified knowledge of the biodegradation mechanism of DPA completed, it is now of further interest to determine the origin of the DPA dioxygenase gene that gives rise to this biodegradation mechanism. Previous research about the mechanism and pathway will help in determining gene origins. To determine the origin of the DPA dioxygenase genes, further knowledge of the sources of the genes is needed. We hypothesize that the DPA dioxygenase gene evolved by gene recruitment from carbazole degrading bacteria through horizontal gene transfer.

We used selective enrichment from the DPA contaminated site at the DuPont Repauno plant in Gibbston, NJ as our primary procedure along with DNA sequencing of PCR amplicons to evaluate similarity of the genes. Several experiments were conducted. The first tested whether bacteria able to grow with carbazole as the sole carbon and nitrogen source can be isolated from the DPA contaminated site. We selectively enriched the bacteria by providing the soil samples with different concentrations of carbazole. The enrichment cultures were established by placing the natural source inoculum into selective media and incubated under appropriate conditions. The inoculum consisted of natural sources such as soil, mud and lake sediment (12). Selective enrichment was done by giving the bacteria carbazole as the only carbon and nitrogen source. The bacteria that were able to grow on the carbazole were selected and isolated. Once carbazole degraders were isolated, the gene that encodes carbazole dioxygenase were amplified using PCR

and specific primers. Our future plan is to have the amplicons sent for sequencing and the resulting sequence will be compared to those of the DPA dioxygenase gene (8).

IV. Principles of isolation

Isolation of specific bacteria is the key step in this experiment. Incubation of soil samples at different concentrations of carbazole as the sole carbon source will lead to selective enrichment of bacteria capable of growing on carbazole.

V. Principles of characterization

HPLC was the main method used to quantify the degradation of carbazole within different culture samples. When the bacteria were growing on carbazole, I had to be able to determine whether or not the concentration of carbazole remained the same or decreased; indicating degradation.

The HPLC specifically provided identification and quantification of carbazole. HPLC was used in this experiment because it is capable of detecting specific chemicals in liquid samples. The bacterial samples were grown in liquid media in microplates.

VI. Principles of identification (Future Plans)

DNA Sequencing and 16S rRNA genes will be used as primary measures to identify the bacteria capable of degrading carbazole. Bacteria is able to degrade carbazole, and once that bacterium has been isolated and purified, the DNA will be sent for sequencing and it will be analyzed through a database program. Comparison between the DNA sequence of the carbazole degrader and the DPA dioxygenase gene will provide molecular level data to determine whether carbazole is the second of the two providers for the origin of DPA Dioxygenase. Similarities between sequences will allow conclusions regarding horizontal gene transfer as a means of evolution of specialized genes. Along with sequencing the carbazole dioxygenase gene using designed primers, characterization of the isolates will be conducted by sequencing of the 16S rRNA gene. The 16S rRNA genes of each sample will be amplified using forward and reverse primers which correspond to, respectively, of the Escherichia coli 16s rRNA sequence (13).

VII. Applications

The purpose of this project is to provide a better understanding and knowledge of the evolution of the DPA biodegradation pathway that has been observed at contaminated sites. We are hypothesizing that the DPA degradation pathway originated from carbazole and aniline degrading bacteria. Knowledge of the origination of the DPA dioxygenase gene will allow further research into the biodegradation of this primary pollutant at manufacturing sites.

The results of this project will increase our understanding of the biodegradation of synthetic chemicals pathway evolution. The practical impact of this research project is that once

we understand the origin of the dioxygenase gene, the insight could enable prediction of evolution of pathways to degrade chemical pollutants other than carbazole, aniline or diphenylamine.

MATERIALS AND METHODS

Isolation of bacteria able to grow on carbazole

Soil and sediment samples were collected from several locations at Repauno (Figure 2, Table 1) and shipped to the laboratory. The samples were suspended in nitrogen-free minimal medium (BLK) and homogenized then centrifuged at minimal speed of 300 rpm for 30 seconds to remove large soil particles. Carbazole was dissolved in methanol at different concentrations, placed in 96-well plates, and the plate was heated to 65ºC to evaporate the methanol. The cultures of the soil samples were added to the microplates in replicates. The culture was incubated under aerobic conditions at room temperature. When the substrates disappeared from the culture, portions (10%, vol/vol) were transferred into BLK containing carbazole crystals (200 µM). Carbazole concentrations in the culture fluids were measured by high-performance liquid chromatography (HPLC) at specific intervals.

Table I. Samples tested from DPA contaminated site.

Sample #	Name	Sample #	Name
1	Comp A1-A5	14	Rep-S-T-G1-1-MC
2	Comp B1-B5	15	GP-62 0-2/1 of 2
3	Comp C1-C5	16	GP-62 0-2/2 of 2
4	Comp D1-D5	17	GP-70 0-2'/1 of 2
5	Comp E1-E5	18	GP-70 0-2'/2 of 2
6	Comp F1-F5	19	GP-61 0-1/1 of 2
7	Comp G1-G5	20	Sediment
8	Rep-S-T-A1-1-MC	21	SWMU8 Tar
9	Rep-S-T-B1-1-MC	22	SWMU8 T+S
10	Rep-S-T-C1-1-MC	23	Anaerobic Sample
11	Rep-S-T-D1-1-MC	24	GP-46 0-1/2 of 2
12	Rep-S-T-E1-1-MC	25	T6-A
13	Rep-S-T-F1-1-MC	26	T10-A

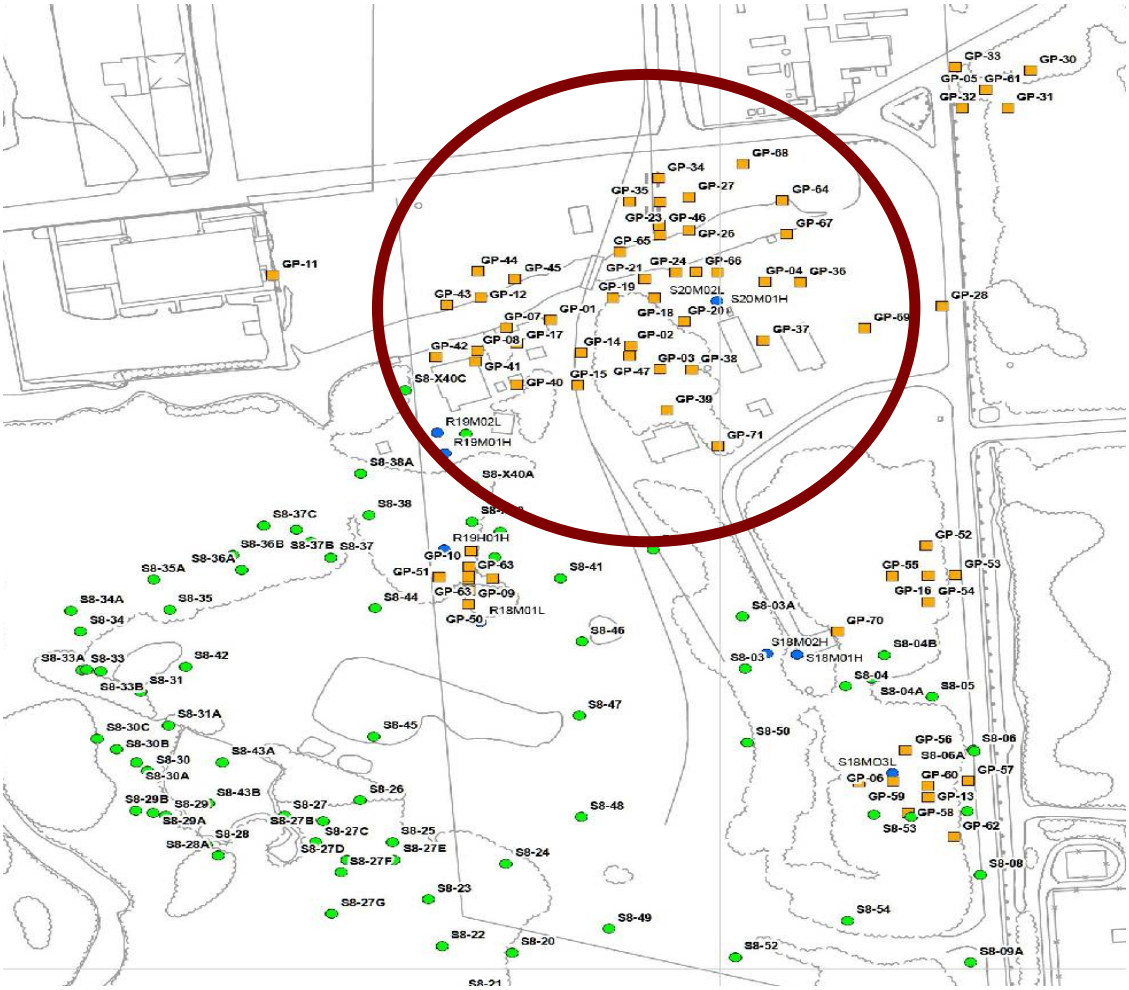

Figure 1. DuPont Repauno plant in Gibbston, NJ. DPA contaminated site.

Growth of Carbazole degraders

Approximately 30 samples from soil were collected and 1.0 g of each sample was suspended in BLK minimal media and homogenized. The homogenized sample was then centrifuged at minimal speed and time to spin down the soil particles to the bottom of the eppendorf tube but maintain free floating bacteria at the top of the sample. The supernatant containing the bacteria was inoculated into a 96-well plate. The 96-well plate contained different concentrations of carbazole ranging from 50 µM carbazole to 500 µM carbazole. The carbazole was dissolved in methanol and dispensed into microplates. The methanol was allowed to evaporate and after inoculation, the bacteria samples were allowed to grow at room temperature in BLK minimal media. Bacteria able to degrade carbazole as a sole carbon and nitrogen source produced turbidity within the microplates signifying bacterial growth. These results from the selective enrichments were used to isolate bacteria capable of degrading carbazole. When it became evident that a culture was able to grow on carbazole as its sole carbon and nitrogen source, I isolated those bacteria individually. The same cultivations were done over 3 generations, and subcultures showing optical density more than 1.0, was spread on the BLK agar (1.8%) medium plates with carbazole and cultivated at room temperate in a dessicator. The BLK agar medium with carbazole contained (per liter of distilled water): 400 µM Carbazole, 27 ml Stainer's solution A (Na2HPO4: 2.75 g/L DI water, KH2PO4: 1 g/L DI water), 1 ml each of solutions B (CaCl2·2H2O: 10 g/L DI water), C (Ferric Citrate: 2 g/L DI water) , D (MgSO4·7H2O: 1.97 g/L DI water) in 970 ml distilled water. The initial pH was adjusted to 7.2. Individual colonies were isolated and purified by repeating isolation of single colonies.

Optimizing Experimental Design via HPLC Verification

Carbazole is insoluble in aqueous media. Because an evenly distributed homogenized mixture of carbazole in a solvent was needed for selective enrichment, carbazole was dissolved in methanol. Methanol was used for several reasons: it has a lower boiling point than carbazole, allowing a more rapid evaporation when exposed to heat, and its ability to dissolve carbazole in the absence of heat or high pressure. To verify that methanol was optimal for this experiment, samples containing carbazole and acetonitrile were run against samples containing methanol and acetonitrile to determine whether the HPLC would produce overlapping peak readings. Under the same HPLC method, methanol and carbazole eluted at different times (Table 2). Along with verifying that methanol and carbazole do not overlap, a standard curve was also constructed. The standard curve composed of different concentrations of carbazole from 50 µL to 400 µL is used to determine specific concentrations of carbazole contained in culture samples at specific elution times (Figure 2).

Table 2. HPLC analysis of carbazole vs. methanol.

Samples	Time [Min] Retention	Area % [%] Peak	Area [mAU.Sec] Peak
500 µL Water	3.97		87.975
500 µL of 400 µM carbazole + methanol with 500 µL acetonitrile	4.25	93	5609.7
500 µL methanol with 500 µL acetonitrile	2.98	63	4.2

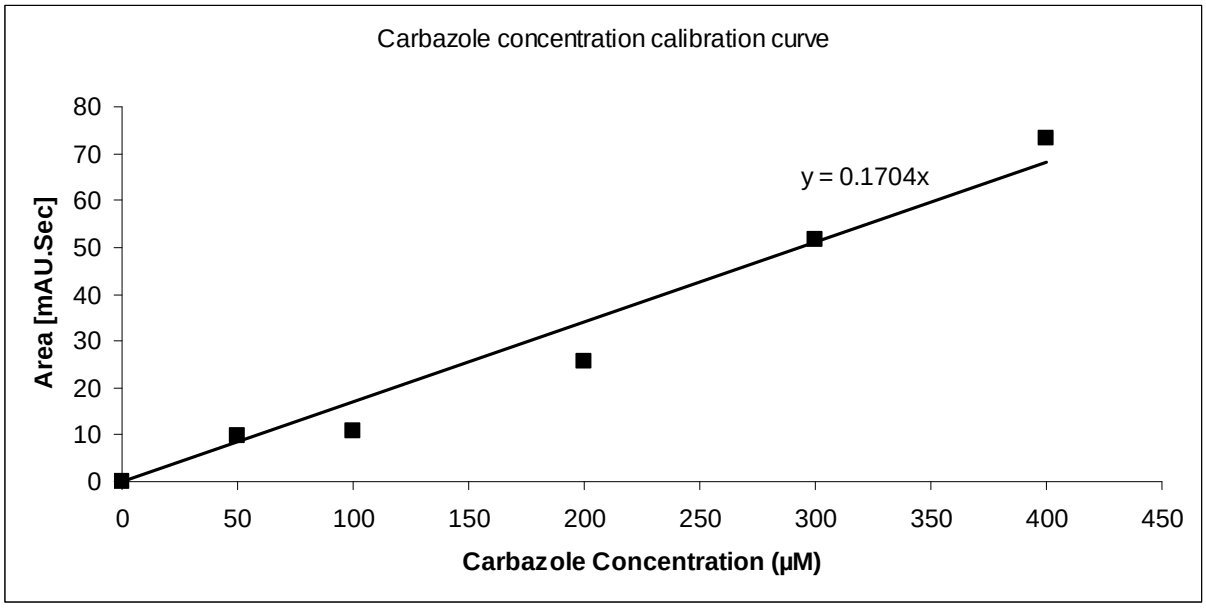

Figure 2. Calibration curve (■ : Carbazole) This curve will be used to calculate and approximate areas of different culture samples at different carbazole concentrations.

Analytical methods

Analytical methods were done using HPLC methods as described in Shin and Spain (8): "Carbazole and its degradation intermediates were separated by paired ion chromatography on a Merck Chromolith RP18e column (4.6 mm by 100 mm) with a Varian HPLC system equipped with photodiode array detector. The mobile phase consisted of part A (5 mM paired ion chromatography low-UV reagent A [Waters, MA] in 20% methanol–80% water) and part B (80% methanol– 20% water). The flow rate was 2 ml/min. The mobile phase was changed from 100% part A to 100% part B over a 2-min period and then held at 100% part B for 2 min. Carbazole was monitored at 200nm."

PCR amplification (Tentative – (8))

Genomic DNA was isolated from carbazole degraders using selective enrichment. Genomic DNA samples (1 ul) were subjected to PCR amplification by the primer pairs shown in Table 3. In order to amplify the gene sequence (1.1 kbp) of carbazole dioxygenase, 30 cycles of amplification were carried out as follows: 95°C for 1 min, 50 °C for 30 s, and 72°C for 1:30 min, after initial denaturation at 95°C for 10 min. PCR products were purified and then sent for sequencing (8).

Table 3. Primers used for amplification of carbazole dioxygenase.

PCR Primers	Description
BORF1-F2	PCR amplification of gene fragment (3014-3035) 5’ AGCAGTCCATCAGAGGTGCTCT-3’
BORF1-R2	PCR amplification of gene fragment (4196-4177) 5’ TGTCCGATAATTTGGAGTCTCT-3’

Tentative Bacteria Identification

“Genomic DNA was extracted with a Genomic DNA purification system (Promega, Madison, WI). The 16S rDNA was PCR amplified with fD1 and rD1 universal primers. PCR products were purified with Wizard® SV Gel clean-up system (Promega, Madison, WI) and sequenced by Nevada Genomics Center (Reno, NV). The resulting 16S rDNA sequences (600 bp) were compared with the sequences in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) using BLAST” (8, 14).

Tentative DNA Sequencing and Sequence Analysis

"The sequences were analyzed with BioEdit 7.0.4. Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA). Sequence databases were searched using the BLAST programs via the National Center for Biotechnology Information website. Multiple sequence alignments were done using ClustalW and phylogenetic analysis was performed by using the neighbor-joining algorithm found in BioEdit. Phylogenetic trees were drawn using the TreeView program"(8).

Degradation of carbazole by DPA degraders

Soil samples containing DPA degraders were from the Repauno site provided by Zuzi Kurt. 6 samples from soil were collected and 1.0 g of each sample was suspended in BLK minimal media and homogenized (Table 1). The homogenized sample was then centrifuged at minimal speed and time to spin down the soil particles to the bottom of the eppendorf tube but maintain free floating bacteria at the top of the sample. The supernatant containing the bacteria were inoculated into a test tube containing 3 mL BLK minimal media with carbazole crystals. The bacteria samples were allowed to grow at room temperature for 48 hours. To test whether a culture was able to grow on carbazole as its sole carbon and nitrogen source, samples were run through the HPLC at the same parameters used to detect carbazole for selective enrichment. Retention time of DPA was similar to the retention time of carbazole. However, DPA eluted at 4.72 min (Table 5). Retention time of DPA and area was used to determine DPA degradation.

Table 4. Soil samples containing DPA degraders

Sample 27	A2 Comp 1
Sample 28	B2 Comp 1
Sample 29	C2 Comp 1
Sample 30	D2 Comp 1
Sample 31	E2 Comp 1
Sample 32	F2 Comp 1
Sample 33	G2 Comp 1
Sample 34	H2 Comp 1

Table 5. HPLC retention time analysis of carbazole and DPA

Samples	Time [Min] Retention
BLK + 400 µM carbazole crystals	4.25
BLK + 400 µM DPA crystals	4.72

Degradation of DPA by carbazole degraders

Carbazole degrader isolates were isolated from the selective enrichment methods. 14 isolates from soil samples were collected and inoculated in BLK minimal media with 400 µM DPA crystals. The bacteria samples were allowed to grow at room temperature for 48 hours. To test whether the culture was able to grow on DPA as its sole carbon and nitrogen source, samples were run through the HPLC at the same parameters used to detect carbazole. Retention time of DPA and area was used to determine whether DPA had degraded or not.

RESULTS

Optimization of carbazole incorporation

Carbazole is soluble in methanol and insoluble in water. Because the experiment required a certain amount of carbazole per sample, carbazole had to be a homogenized solution. BLK minimal media was used due to its absence of carbon and nitrogen – both sources which may be used by bacteria to grow. Carbazole had to be dissolved into the minimal media because carbazole was provided in solidified form. The solution that we used to dissolve carbazole was methanol. Carbazole (1 mM) was dissolved in 10 mL of methanol to produce a homogenized solution – no heat was added. To quantitatively check whether methanol was a proper solvent, control systems were tested through the HPLC. A solution consisting of carbazole and methanol was tested against methanol alone. HPLC results indicated that methanol and carbazole eluted at different times and did not coincide with each other (Table 2). Therefore, methanol was successfully used to dissolve carbazole and evaporating the methanol before preparations into the multi-well plate was sufficient to remove all methanol from the microplates.

Primary Screening: Which samples degrade carbazole

The question for the screening method was to determine which soil sample was able to degrade carbazole. Using the enrichment method, we saw that samples from the Repauno site were able to degrade carbazole as the sole carbon, nitrogen and energy source. Therefore, carbazole degrading bacteria was successfully isolated from the DPA contaminated site and provided evidence that carbazole degraders were present.

Table 6. Initial and final concentration of select carbazole degraders after 48 hours of inoculation.

Sample	**Initial [carbazole] (µM)**	**Final [carbazole] (µM)**
Rep-S-T-E1-1-MC	447.77	22.89
Rep-S-T-D1-1-MC	424.30	1.17
Sediment	428.99	4.11
Comp B1-B5	406.10	5.28
Comp A1-A5	490.61	27.00

Serial Dilutions of Carbazole Degrading Bacteria

For all the positive bacteria that were capable of degrading carbazole based on the HPLC results, serial dilutions were done. Serial dilutions were done up to 6 dilutions of ten times. Each well contained a constant concentration of carbazole dissolved in methanol. Soil samples that were shown to be capable of degrading carbazole using the HPLC were recorded. Of all the samples that were tested using serial dilutions, only sample 7, Comp G1-G5 had a decreasing trend as the dilutions got higher shown in the row labeled with "sample 7" with the bolded optical density value of 0.441. This was shown not only though physical appearance of turbidity in the 96-well plate but also though the absorbance measurement of optical density (Figure 4, Table 7). The values of all the samples that were able to degrade carbazole tested through serial dilution have an unusual upward trend in growth and optical density.

Table 7. Soil sample 7 and absorbance readings at 600nm. Sample 7 was the sample that showed positive results and was able to grow consistently through serial dilution of ten times.

Serial Dilution (10x Dilutions)		1	2	3	4	5	6
Sample 1	A	0.441	0.866	0.998	1.027	1.057	1.241

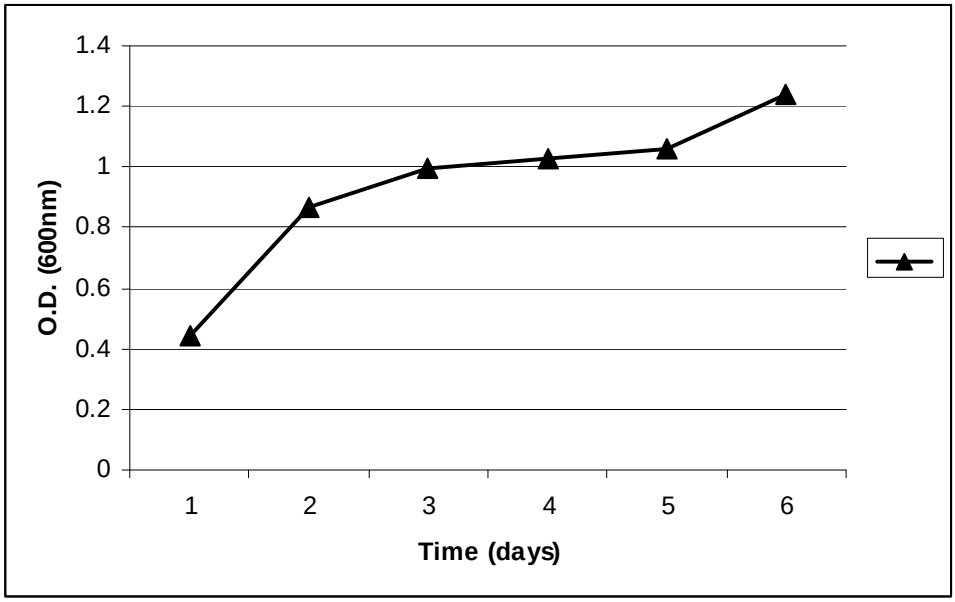

Figure 12. Growth of carbazole degrader 7 (comp G1-G5) on carbazole as sole carbon and nitrogen source. (▲: Optical density at 600 nm)

DPA degrading bacteria: carbazole degradation

DPA degrading bacteria were tested to determine whether they had the capabilities to degrade carbazole. DPA contaminated soil was provided by Repanuo. The DPA degraders were tested with carbazole as the sole carbon, nitrogen and energy source. After 48 hours of incubation at room temperature, the concentration of carbazole was measured using the HPLC. Results show that DPA degrading bacteria were not capable of degrading carbazole (Table 8).

Table 8. DPA degraders inoculated with carbazole (400 µM) Final concentration of carbazole after 48 hours of incubation at room temperature

Sample	Final [Carbazole] µM
Control: BLK + Carbazole	400
H2-1-M-Comp (0-0.5)	438
G2-1-M-Comp (0-0.5)	470
F2-1-M-Comp (0-0.5)	758
E2-1-M-Comp (0-0.5)	290
D2-1-M-Comp (0-0.5)	389
B2-1-M-Comp (0-0.5)	713

Carbazole degrading bacteria: DPA degradation

Carbazole degrading bacteria were tested to determine whether they had the capabilities to degrade DPA. Using carbazole degraders from the selective enrichment experiment, the isolates were grown with DPA as the sole carbon, nitrogen and energy source. After 48 hours of incubation at room temperature, the concentration of DPA was measured using the HPLC. Results show that carbazole degrading bacteria were/were not able to degrade DPA (Table 9).

Table 9. Carbazole degraders inoculated with DPA (400 µM) Final concentration of DPA after 48 hours of incubation at room temperature.

Sample	Final [DPA] µM
Control: BLK+DPA only	400.00
D2-1-M-Comp (0-0.5)	386.85
GP 70 0-2/2 of 2	324.10
GP 70 0-2/1 of 2	360.92
SWMU 8 Tar+Soil	402.27
REP-S-T-G1-1-MC	383.89
REP-S-T-E1-1-MC	361.58
REP-S-T-D1-1-MC	341.66
Comp G1-G5	302.68
Comp F1-F5	277.43
Comp E1-E5	304.19
Comp D1-D5	388.57
Comp C1-C5	301.59
Comp B1-B5	369.92
Comp A1-A5	397.35

CONCLUSION

We tested a number of samples that were provided from a DPA contaminated site. The experiment was to attempt to determine whether or not the DPA dioxygenase came from carbazole dioxygenase from Repauno. The soil samples were selectively enriched to test whether or not they were able to degrade carbazole. Serial dilution results showed that sample 7, Comp G1-G5, was the only sample capable of consistently using carbazole as its carbon source. With sample 7 we will perform a PCR using forward and reverse primers for amplification of the carbazole dioxygenase gene. The amplified carbazole dioxygenase gene will be compared to the dioxygenase gene and the identity of sample 7 will be identified by sequencing the 16s rRNA sequence.

The second part of the experiment was to determine whether or not carbazole degrading bacteria would be able to degrade DPA and whether or not DPA degrading bacteria would be able to degrade carbazole. The reason for testing this was to determine whether carbazole degrading bacteria provided DPA degrading bacteria with DPA dioxygenase. The hypothesis was that the pathways did not coexist. DPA degrading isolates were tested with carbazole. In conjunction with testing DPA degrading isolates with carbazole, we also tested carbazole degraders with DPA as their sole carbon, nitrogen and energy source. The results of this experiment showed that carbazole degraders did not degrade DPA.

To further support our hypothesis that DPA dioxygenase evolved through horizontal gene transfer, we tested whether or not DPA degraders would be able to degrade carbazole. We assumed that the DPA degraders would not be able to degrade carbazole due to our evolution hypothesis that DPA dioxygenase originated from the gene transfer between carbazole and

aniline degrading bacteria. Our data shows that DPA degrading isolates were not able to degrade carbazole. This is further evidence that the DPA degrading bacteria only has the DPA dioxygenase and not the rest of the pathway. This is advantageous for the bacteria because the pathways for DPA degradation and carbazole degradation will not coincide with each other. A possible reason why the degraders did not grow could be that the metabolites for one pathway could be toxic for the other pathway causing misrouting. Coinciding degradation pathways can not only be detrimental but also cause misrouting of intermediates. In the development of DPA dioxygenase, aniline degraders had to have undergone horizontal gene transfer with another source to have formed the DPA dioxygenase. We hypothesize that the other contributor is the carbazole degrader. Therefore we are comparing the carbazole dioxygenase with the DPA dioxygenase in further studies to determine whether DPA dioxygenase has similarities with carbazole dioxygenase. This similarity in genetic sequence between the two dioxygenases will allow us to make the conclusion that DPA dioxygenase developed evolutionarily from aniline and carbazole degrading bacteria at Repauno.

FUTURE STUDIES

PCR will be used to amplify the carbazole dioxygenase gene and the amplified gene sequence will be sent out for sequencing. Once the sequencing comes in, comparison between the DPA dioxygenase gene and the carbazole dioxygenase gene will be made. Comparison between the 2 genes will indicate whether or not horizontal gene transfer occurred to create the DPA dioxygenase gene.

Along with purifying the genomic DNA for the carbazole dioxygenase gene, other samples including aniline degrading bacteria will be tested. The purified isolates of aniline degrading bacteria are candidate samples if the bacteria are able to not only degrade aniline but also be able to degrade carbazole, it will support the hypothesis that the DPA dioxygenase gene was created through horizontal gene transfer between 2 genes.

While serial dilutions are being grown, carbazole degrading methods are also being optimized. The samples that are being used to optimize the approach are carbazole degraders provided by Kwanghee Shin (Table 1). The stocks will be streaked onto non-selective plates such as TA, LB or a form of nutrient broth to allow growth. The non-selective plates will not contain carbazole and the stocks will be allowed to form colonies. Once colonies form, they will be transferred to carbazole plates that contain a concentration of carbazole of 400 µM, consistent with the carbazole concentration used for the glycerol isolates. The stocks will be allowed to grow until colonies form on the carbazole agar plates and the success of the growth will be tracked through either visual judgment of looking for present colonies on agar plates or measured numerically by measuring optical density if transferred to liquid media.

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