Metabolic Engineering to Develop a Pathway for the Selective Cleavage of Carbon-Nitrogen Bonds

ANNUAL TECHNICAL REPORT

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ABSTRACT

The objective of the project is to develop a biochemical pathway for the selective cleavage of C-N bonds in molecules found in petroleum. Specifically a novel biochemical pathway will be developed for the selective cleavage of C-N bonds in carbazole.

The cleavage of the first C-N bond in carbazole is accomplished by the enzyme carbazole dioxygenase, that catalyzes the conversion of carbazole to 2-aminobiphenyl-2,3-diol. The genes encoding carbazole dioxygenase were cloned from *Sphingomonas* sp. GTIN11 and from *Pseudomonas resinovorans* CA10. The selective cleavage of the second C-N bond has been challenging, and efforts to overcome that challenge have been the focus of recent research in this project. Enrichment culture experiments succeeded in isolating bacterial cultures that can metabolize 2-aminobiphenyl, but no enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl has been identified. Aniline is very similar to the structure of 2-aminobiphenyl and aniline dioxygenase catalyzes the conversion of aniline to catechol and ammonia. For the remainder of the project the emphasis of research will be to simultaneously express the genes for carbazole dioxygenase and for aniline dioxygenase in the same bacterial host and then to select for derivative cultures capable of using carbazole as the sole source of nitrogen.

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EXECUTIVE SUMMARY

The objective of the project is to develop a biochemical pathway for the selective cleavage of C-N bonds in molecules found in petroleum. Specifically a novel biochemical pathway will be developed for the selective cleavage of C-N bonds in carbazole. This is the second annual report in a 3-year research project and encompasses the period from October, 2004 to September, 2005.

The development of biocatalysts with improved ability to cleave carbon-nitrogen bonds is being addressed by cloning the genes for carbazole dioxygenase and aniline dioxygenase. The removal of nitrogen from aromatic compounds like carbazole requires the cleavage of two carbon-nitrogen bonds. The cleavage of the first carbon-nitrogen bond is accomplished by the enzyme carbazole dioxygenase while an enzyme capable of selectively cleaving the second carbon-nitrogen bond has not yet been identified. The enzyme carbazole dioxygenase is encoded for by three genes: *carAa, carAc*, and *carAd*. The *carAacd* genes have been cloned and sequenced from several different microbial cultures including *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10. While an enzyme that can selectively cleave the second carbon-nitrogen bond in carbazole has not yet been identified a reasonable candidate for such an enzyme is the aniline dioxygenase from *Acinetobacter* sp. YAA. The genes that encode aniline dioxygenase (*atdA1, atdA2, atdA3, atdA4*, and *atdA5*) have been cloned. These genes will be expressed in *Rhodococcus erythropolis*, a bacterial host that tolerates exposure to petroleum. Gene expression vectors for use in *Rhodococcus erythropolis* are being constructed from plasmids pEBC26, pSRKgfp, and pRESQ.

Enrichment culture experiments designed to isolate a culture capable of cleaving the carbon-nitrogen bond in 2-aminobiphenyl, and thus being capable of providing an enzyme for the cleavage of the second carbon-nitrogen bond in carbazole, resulted in the isolation of a unique microbial culture *Pseudomonas* sp. GTIN-G4. *Pseudomonas* sp. GTIN-G4 is capable of metabolizing 2-aminobiphenyl and related compounds, but does not appear to be capable of cleaving the carbon-nitrogen bond. Instead it has the unprecedented ability to modify organonitrogen compounds by replacing a hydrogen bound to the nitrogen atom with a formaldehyde group. This is a very interesting metabolic ability, but it does not allow the removal of nitrogen from organonitrogen compounds so this culture will not be examined further.

The time remaining in this project does not allow for continued enrichment culture experiments seeking to identify an enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl. Accordingly, future experiments will utilize enzymes/genes that are cloned and available to the project, and known to be capable of selectively cleaving C-N bonds in substrates that are chemically similar to carbazole and its metabolites. The focus of research will be on constructing a new metabolic pathway for the selective cleavage of C-N bonds by expressing the genes for carbazole dioxygenase and for aniline dioxygenase in the same bacterial host, *Rhodococcus erythropolis*. Subsequent selection for growth under conditions where carbazole serves as the only source of nitrogen will hopefully result in the isolation of a novel biochemical pathway for the selective cleavage of C-N bonds in carbazole.

INTRODUCTION

With the decline in the production of light and medium weight crude oils, refineries will increasingly be forced to process heavier and sour crudes. These crude oils are high in sulfur, nitrogen and metals. Nitrogen and sulfur in petroleum can foul catalysts decreasing the efficiency of hydrotreating and catalytic cracking processes. Sulfur-containing compounds typically found in petroleum that are recalcitrant to removal by hydrotreating are dibenzothiophene and alkylated derivatives of dibenzothiophene. The heavier gas oils and residua contain both basic and non-basic nitrogen compounds. The basic nitrogen compounds include pyridine, quinoline, acridine, phenanthridine, and their derivatives. These are responsible for poisoning of FCC catalysts by the reaction of the basic compounds with the acidic sites of the catalyst. The non-basics are predominantly mixed alkyl derivatives of carbazole and account for 70-75% of the total nitrogen content of crude oil (0.3% N). The neutralization of the active acid sites results in deactivation of the catalyst. Nitrogen poisoning also affects the selectivity of the reaction. Carbazole, a major constituent of the non-basic portion (and hence of the total nitrogen present), gets converted into basic derivatives during the cracking process and adsorbs and poisons the catalyst as described above. Nitrogen compounds in petroleum foul catalysts and thus decrease the efficiency of the existing hydrotreating and catalytic cracking processes. In addition to catalyst fouling, nitrogen compounds also promote corrosion of the equipment. Also, the combustion of nitrogen compounds leads to formation of nitrogen oxides (NOx) which, in the presence of other hydrocarbons (VOCs:volatile organic compounds) and sunlight lead to ozone formation. Both ozone and NOx are hazardous to human health. Removal of these organonitrogen compounds will not only significantly improve the efficiency of the catalytic cracking process and result in cost savings for the refinery but also decrease atmospheric pollution. The selective removal of nitrogen from petroleum is a relatively neglected topic in comparison with sulfur removal. Moreover, most metals in oil are associated with nitrogen compounds, and nitrogen compounds contribute to the instability of petroleum byproducts[5, 13, 31]. The selective removal of nitrogen from oil would be highly desirable, but effective processes are not currently available.

There is hence a need to develop alternate cost-effective and energy-efficient technologies for the removal of sulfur, nitrogen and metals. Existing thermochemical processes, such as hydrodesulfurization, can efficiently remove much of the sulfur and nitrogen from

petroleum but the selective removal of all organically bound sulfur and nitrogen, and the removal of metals cannot be efficiently accomplished using currently available technologies. The specificity of biochemical reactions far exceeds that of chemical reactions. Moreover biorefining can be performed at comparatively low temperatures and pressures and does not require hydrogen thus avoiding a significant amount of operating costs associated with the conventional hydrodesulfurization process. The selective removal of sulfur from dibenzothiophene and from petroleum by biochemical reactions performed by microorganisms has been demonstrated, but improved cultures are needed before a viable biodesulfurization process can be developed. Biorefining can also potentially be used to remove nitrogen and metals from petroleum, but so far this area of research has received very little attention.

Biorefining can complement existing technologies by specifically addressing compounds/contaminants refractory to current petroleum refinery processes. Heteroatoms such as nitrogen, metals, and sulfur can poison the catalysts used in catalytic cracking and hydrotreating processes[8, 12, 24, 31]. Existing refineries are not capable of operating efficiently with heavy crude oils and residuum that have high heteroatom content. Bioprocesses could be used to pre-treat oil reducing the heteroatom content allowing the use of heavy crude oils that could not otherwise be treated with existing refinery processes. Biorefining processes can also be used in conjunction with existing processes to meet the increasingly stringent environmental requirements for contaminant reduction. Additionally, most current technologies focus on the removal of sulfur while the development of processes to remove nitrogen, and its associated heavy metals, is a comparatively neglected research topic that will increase in importance as the quality of available petroleum declines[14].

There is currently no biochemical pathway, or thermochemical process, for the selective removal of nitrogen from compounds typically present in petroleum[8, 31]. Previous research by GTI characterized the biochemistry and the genetics of microbial enzymes capable of cleaving one of the two carbon-nitrogen bonds in carbazole[16]. This project extends that work by constructing a biochemical pathway enabling the selective and complete removal of nitrogen from carbazole and related compounds. Thus the successful completion of the project will provide a previously unavailable treatment option for the up-grading of petroleum. Moreover, demonstrating the construction of a novel biochemical pathway will guide future research in overcoming other obstacles for which no technically viable approach is currently available.

Carbazole is a good model compound that is representative of the nitrogen-containing compounds present in the greatest abundance in many petroleum samples[5, 13, 19]. For developing a biological process for the removal of nitrogen from petroleum no known carbazole-degrading culture is particularly appropriate because nitrogen is only removed in the course of overall degradation[2, 11, 16-18, 20, 23, 25, 29, 30]. A microorganism capable of selectively cleaving C-N bonds in quinoline and removing nitrogen from petroleum was isolated and characterized[15] (by GTI). But quinoline is not the predominant form of organonitrogen compounds in petroleum. What is wanted is selective cleavage of both C-N bonds in carbazole, and related compounds, resulting in the selective removal of nitrogen while leaving the rest of the molecule intact.

A variety of carbazole-degrading microorganisms have been reported in the literature including *Sphingomonas*, *Pseudomonas, Mycobacterium, Ralstonia* and *Xanthamonas* species[2, 11, 16-18, 21, 26, 27, 29, 30]. Insofar as biodegradation pathways have been investigated, these differing species of carbazole degraders follow a similar carbazole degradation pathway that begins with the oxidative cleavage of the heterocyclic nitrogen ring of carbazole to form 2'-aminobiphenyl-2,3-diol. This compound is then oxidized through meta cleavage yielding 2-hydroxy-6-oxo-6-hexa-2e,4z-dienoate. The next metabolic steps result in the degradation of one of the aromatic rings releasing carbon dioxide. In existing pathways nitrogen is released from carbazole only after substantial carbon degradation. Figure 1 illustrates the carbazole degradation pathway employed by all currently known carbazole utilizing cultures as well as the pathway for selective removal of nitrogen from carbazole that will be created in this project.

Therefore several bacterial cultures are known that can utilize carbazole as a sole nitrogen source, but no culture is known that can selectively cleave both C-N bonds in carbazole while leaving the rest of the molecule intact.

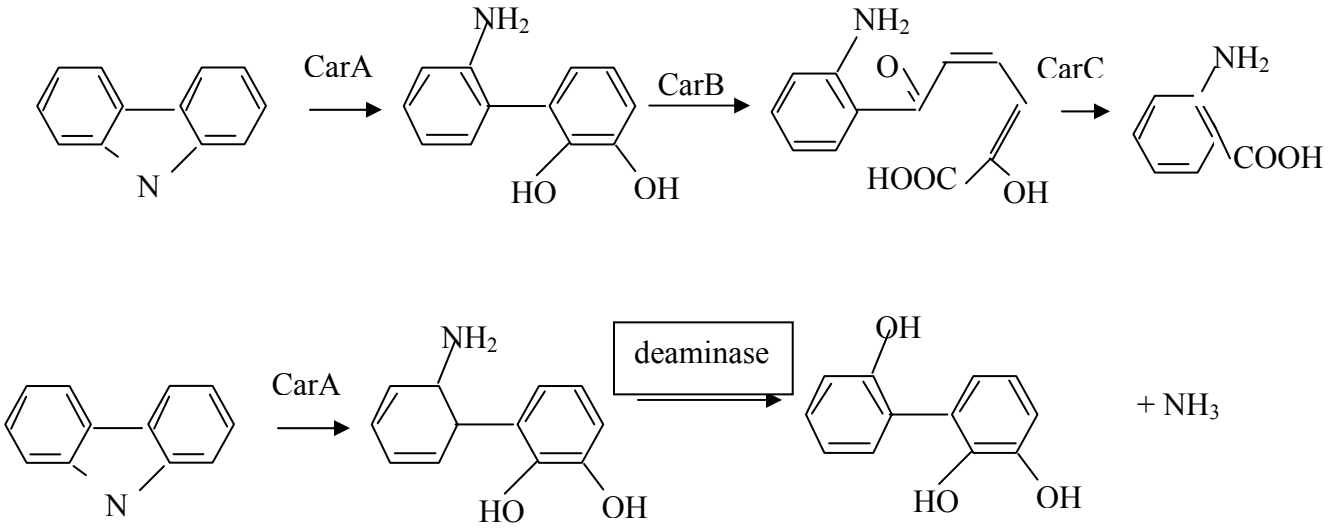


FIGURE 1. CARBAZOLE DEGRADATION PATHWAYS.

The top pathway illustrates the existing carbazole degradation pathway that results in overall degradation, whereas the bottom pathway illustrates the pathway for the selective removal of nitrogen from carbazole that will be developed in this project.

Sphingomonas sp GTIN11 [16]was isolated by GTI scientists and demonstrated to metabolize carbazole, and to a lesser extent C1 and C2 derivatives of carbazole, from petroleum. Moreover, the genes encoding a portion of the carbazole degradation pathway of *Sphingomonas* sp. GTIN11 have been cloned and sequenced. The reaction catalyzed by CarA converts carbazole to 2'-aminobiphenyl-2,3-diol accomplishing the cleavage of the first C-N bond in carbazole. There are no known deaminases that can metabolize 2'-aminobiphenyl-2,3-diol and accomplish the cleavage of the final C-N bond[7, 9]. This project will use enrichment culture, and directed evolution to isolate and/or create a deaminase that will recognize 2'-aminobiphenyl-2,3-diol as a substrate. The gene encoding an appropriate deaminase will be identified, sequenced, and combined with the *carA* genes (*carAa, carAc*, and *carAd* encoding for the

carbazole dioxygenase, ferredoxin and ferredoxin reductase respectively) from *Sphingomonas* sp. GTIN11 and thereby construct a synthetic operon for the selective removal of nitrogen from carbazole, as shown in Figure 1. The *carA* genes from *Sphingomonas sp*. GTIN11 will be used in the proposed work because this is the only carbazole degrading culture demonstrated to remove nitrogen from petroleum. A preferred bacterial strain would lack the *carB* and *carC* genes[11, 25, 29] so that complete biodegradation of carbazole would be avoided and the final product would be 2',2,3-trihydroxybiphenyl (or a similar compound).

Enrichment culture experiments will be performed to isolate bacterial cultures capable of utilizing 2-aminobiphenyl as a sole nitrogen source. Cultures will then be tested to determine if they contain an enzyme that can deaminate 2-aminobiphenyl. Because 2'aminobiphenyl-2,3-diol is not commercially available enrichment culture experiments will employ 2-aminobiphenyl. Known deaminases have limited ability to metabolize aromatic amides and their substrate range includes benzamide, toluamide, and anthranilamide[7, 9]. However, these enzymes can possibly by modified to recognize 2-aminobiphenyl as a substrate. Alternatively, the initial step in aniline degradation, catalyzed by aniline dioxygenase, is the conversion of aniline to catechol and ammonia. The chemical similarity between aniline and 2-aminobiphenyl make aniline dioxygenase a good candidate enzyme for potentially cleaving the C-N bond in 2-aminobiphenyl. Once bacterial cultures are available that can deaminate 2-aminobiphenyl, then the deaminase gene will be cloned, sequenced, and combined with the *carA* genes of *Sphingomonas* sp. GTIN11 to create a novel metabolic pathway for the selective cleavage of C-N bonds. It will be verified that the newly constructed pathway confers the ability to selectively cleave both C-N bonds in carbazole.

This project is relevant to the DOE's interests because it can contribute to greater efficiency in petroleum refining and can decrease environmental pollution due to the use of petroleum. Nitrogen in petroleum contributes to air pollution and decreases refinery efficiency by poisoning catalysts, but it is difficult to remove organically bound nitrogen without destroying the calorific value of the fuel. A key objective of the project is to develop biochemical pathways for the selective cleavage of C-N bonds in molecules found in petroleum. The successful completion of this project will enable the development of a bioprocess to selectively remove nitrogen, and associated metals, from crude oil and residuum which will allow existing. refineries to process lower quality oils than they could not otherwise accept. The reduction of

nitrogen and metals in petroleum will allow refineries to operate more efficiently. This will decrease costs and will protect the environment[24].

In North America alone over 3 trillion barrels of known petroleum reserves are largely untapped or underutilized because of their high sulfur/nitrogen/metals content and attendant viscosity problems[32]. Energy statistics indicate that the U.S. imports 65% of its oil demand[14]. New technologies, such as the proposed work, will allow a greater utilization of heavy oils and residuum while still maintaining refinery efficiency and environmental protection. The National Petroleum Refineries Association estimated the cost of meeting Clean Air Act regulations requiring a maximum sulfur content of 0.05% for diesel fuel by 1994 cost about $3.3 billion in capital expenditures and $1.2 billion in annual operating costs[14, 24]. Similar estimates for the removal of nitrogen and metals from heavy oils and residuum are not available. However, diesel is far easier to treat than heavy oils so that one would predict that the costs associated with upgrading heavy oils and residuum would be correspondingly higher. The removal of nitrogen and metals prior to combustion of petroleum also protects the environment by eliminating contaminants that would otherwise contribute to air pollution.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

Environmental samples were obtained from petroleum and/or hydrocarbon contaminated soil. The environmental samples were used to inoculate nutristat and shake flask directed evolution/enrichment culture experiments to obtain cultures that may be suitable for the metabolism of organonitrogen compounds. A further description of the methodologies used in the isolation and characterization of bacterial cultures that can selectively cleave C-N bonds can be found in recent publications by GTI: Kilbane II, J. J., A. Daram, J. Abbasian, and K. J. Kayser, 2002, "Isolation and characterization of *Sphingomonas* sp. GTIN11 capable of carbazole metabolism in petroleum" Biochemical & Biophysical Research Communications 297: 242-248, and Kilbane II, J. J., R. Ranganathan, L. Cleveland, K. J. Kayser, C. Ribiero, and M. M Linhares, 2000, "Selective removal of nitrogen from quinoline and petroleum by *Pseudomonas ayucida* IGTN9m", Applied & Environmental Microbiology 66: 688-693.

Multiple nutristats were set up employing a defined nitrogen-free mineral salts medium (Mod A):

KH_2PO_4	0.37 g/L
$MgSO_4.7H_2O$	0.25 g/L
$CaCl_2.2H_2O$	0.07 g/L
$FeCl_3$	0.02 g/L
Glucose/glycerol/succinate	20.0 g/L

This medium was adjusted to pH 6.5 to 7 and nitrogen was supplied in the form of an organonitrogen test compound in the 0.5-20 mM concentration range. For the positive nitrogen control, 10 mM NH_4Cl (0.535 g/L) was used.

An improved recipe for a defined mineral salts media, MMN, that yielded better growth of some bacterial isolates was also used. A mixture of glucose, glycerol, and succinate was employed as a carbon source to encourage the growth of a wide range of microbial species. The recipe of the improved nitrogen-free minimal media MMN is:

Compound Name	1 X
EDTA	3.2 mg
MOO_3	0.1 mg
Na_2HPO_4	1,419.6 mg
$KH_2\ PO_4$	1,360.9 mg
$MgSO_4$	98.5 mg
$CaCl_2 \bullet 2H_2O$	5.88 mg
H_3BO_4	1.16 mg
$FeSO_4 \bullet 7H_2O$	2.78 mg
$ZnSO_4 \bullet 7H_2O$	1.15 mg
$MnSO_4 \bullet H_2O$	1.69 mg
$CuSO_4 \bullet 5H_2O$	0.38 mg
$CoCl_2 \bullet 6H_2O$	0.24 mg

*** In 1 L ddH_2O

Nutristats and shake flasks were operated at temperatures of 25 (room temperature), 37, and 45 °C. The working volume of nutristats is one liter and shake flask experiments generally utilize 25 to 100 mL of liquid medium. The organonitrogen test compound is routinely varied during the course of operation of the nutristats/shake flask experiments. Nutristats are operated in series so that the effluent of one nutristat serves as the influent for the next. Carbazole, 2-aminobiphenyl, benzamide, aniline, 4,4'-azodianiline, ortho-, meta-, and para-toluidine, quinoline, pyridine, quinazoline, quinoxaline, piperidine, pyrolidine, triazine or other test compounds are added to the fresh media influent at concentrations of 0.5-20 mM. Flow rates of the nutristats are adjusted to achieve hydraulic retention times ranging from 35 hours to 60 hours. The flow rates and the organonitrogen test compound are altered as needed to ensure that the nutristats create an environment suitable for the selection of cultures with improved abilities to selectively cleave C-N bonds. This means that the bacterial cell density in the nutristats/shake flasks ranges from 10^2 to 10^8 cells/mL, but generally cell densities of 10^4 to 10^5 cells/mL are maintained. The bacteria isolated from the effluent of nutristats and/or from shake flasks or nitrogen bioavailability assays are subjected to short wave ultraviolet (UV) irradiation. Cell populations are mutagenized under conditions that result in the death of about 99% of the population. The mutagenized cells are then used to reinoculate nutristats, start additional shake flask experiments, and to streak onto agar plates containing organonitrogen test compounds. Care is taken to ensure that the amount of biomass that is added back to nutristats in the form of inocula is insufficient to provide a significant amount of nitrogen in the form of dead biomass.

Hence significant bacterial growth in the nutristat experiments should be due to the utilization of nitrogen from the organonitrogen test compounds and not from readily available sources such as dead biomass. The effluent of nutristats and cells from shake flasks and from agar plates are routinely tested using the nitrogen bioavailability assay.

For the growth of desulfurization-competent bacterial cultures media that is deficient in sulfur was used. Two recipes for sulfur-free microbial growth media are given below.

	BSM	**MK1**
Na_2HPO_4	5.57 g/L	4.00 g/L
KH_2PO_4	2.44	4.00
NH_4Cl	2.00	2.00
$MgCl_2$-$6H_2O$	0.20	0.20
$CaCl_2$-$2H_2O$	0.001	0.001
$FeCl_3$-$6H_2O$	0.001	0.001

Nitrogen Bioavailability Assay

The nitrogen bioavailability assay utilizes defined mineral salts medium in growth tests in which organonitrogen model compounds such as 2-aminobiphenyl, benzamide, aniline, 4,4'-azodianiline, diphenylamine, maleimide, ortho-, meta-, and para-toluidine, quinoline, pyridine, carbazole, quinazoline, piperidine, pyrolidine, and triazine serve as sources of carbon and/or nitrogen. For selective cleavage of carbon-nitrogen bonds, a culture should be capable of utilizing an organonitrogen compound as a nitrogen source but not as a carbon source. Accordingly, growth tests are performed using the following eight conditions:

1. Test compound as sole source of carbon and nitrogen.
2. Test compound as sole source of carbon (alternative nitrogen source, ammonia, is available).

3. Test compound as sole source of nitrogen (alternative carbon source, glucose/glycerol/succinate, is available).
4. Test compound present as well as alternative sources of carbon and nitrogen.
5. Only alternative nitrogen (ammonia) and carbon (glucose/glycerol/succinate) sources are available. The test compound is not present.
6. No carbon or nitrogen compounds of any kind are present.
7. Only alternative nitrogen (ammonia) is present. No carbon or test compound is present.
8. Only carbon (glucose/glycerol/succinate) sources are available. No nitrogen compounds (ammonia or test compound) are present.

These eight growth conditions constitute a bioassay for the ability of a culture to metabolize organonitrogen compounds. The basis of the nitrogen bioavailability assay is that all microorganisms require nitrogen for growth. When carbon and nitrogen sources other than the test compounds are needed, they will be supplied in the form of glucose/glycerol/succinate, and as ammonia respectively.

The nitrogen bioavailability assay described above can be performed with any organonitrogen test compound that is ordinarily used at a concentration of from 0.5 to 20 mM. The various cultures to be tested are inoculated into test tubes or shake flasks containing medium components appropriate for the eight test conditions. The cultures are then incubated aerobically for 2 to 28 days, at 25, 37, and 45°C. The growth of the cultures is monitored easily by measuring the turbidity/optical density of the cultures in the various test conditions, or by determining colony-forming units. The unamended sample (test condition No. 6) serves as a negative control while the samples amended with both a carbon and nitrogen source (test conditions No. 4 and 5) serves as positive controls and should produce healthy microbial growth unless the test compound is toxic to the culture being tested. If the test compound is toxic only condition No. 5 should result in healthy growth. The amount of bacterial growth observed in test conditions 1, 2, and 3 in comparison with the amount of growth observed in test conditions 4, 5 and 6, indicate the ability of cultures to use the organonitrogen test compound as a source of carbon and/or nitrogen. Those cultures which show better growth in test condition No. 3 than conditions Nos. 1 or 2 may be preferentially utilizing the organonitrogen compound as a nitrogen

source only, and should be examined more thoroughly and included in further experiments. Conditions 7 and 8 serve as negative controls, and no growth should occur unless there is a problem with the batch of media used, or if the bacterial culture being tested has the ability to fix atmospheric nitrogen.

The ability of bacterial cultures to utilize organosulfur compounds as sole sources of sulfur needed for growth can be tested in a Sulfur Bioavailability Assay. It is performed in a manner similar to that described above for the Nitrogen Bioavailability Assay, but employs the sulfur-free media BSM or MK1.

Thin Layer Chromatography for Identification of Metabolites

Thin layer chromatography (TLC) was performed on Whatman Silica C-18 plates by the method described by Watson and Cain (Biochem. J. 146: 157-172, 1975). Running phase solvents used were chloroform-toluene (1:3), and hexane-acetic acid-xylene (5:1:2). Supernatants from bacterial cultures grown with an organonitrogen test compound as the sole source of nitrogen were obtained after centrifugation at 10,000 x g for 15 minutes. These supernatants were used at neutral or alkaline pH. Typically 10 ml of aqueous supernatant was acidified to pH 1 to 2 with HCl and extracted with ethyl acetate (1:1 or 1:0.5 v/v). The organic phase was separated from the aqueous phase by centrifugation or by using a separatory funnel. The ethyl acetate extract was then evaporated in a hood resulting in the concentration of the sample from 20 to 1000-fold prior to the analysis of the extracts by TLC. 10 to 50 μL of ethyl acetate sample that had been concentrated 100-fold relative to the volume of aqueous supernatant extracted was spotted onto TLC plates. Typical running times of the TLC plates were about 20 minutes. These plates were later observed under normal lighting, short (245 nm), and long wave (366 nm) UV light.

Some experiments also utilized resting cells that were prepared by centrifuging from 500 ml of log phase cultures grown with either an organonitrogen compound or ammonia as nitrogen sources. Then the washed cell pellets were resuspended in 5 to 50 ml of mineral salts medium achieving final cell densities of from 10^{10} to 10^{11} cells/ml. These cell suspensions were incubated with from 1 to 20 mM test compound (organonitrogen compound) for periods ranging from 15 minutes to 24 hours. The incubator was agitated at about 200 rpm and maintained at the

microorganism's optimum temperature. The ethyl acetate extract was stored in amber vials at 4°C until they were analyzed by TLC, HPLC and/or GC-MS.

Gas Chromatography-Mass Spectrometry

GC-MS analysis was performed on extracts derived from growing and resting cell cultures exposed to organonitrogen test compounds, and on compounds eluted from spots observed on thin layer chromatography plates.

Extraction of the supernatants from resting cells as well as growing cells were carried out either by ethyl acetate solvent extraction or with C-18 solid phase extraction cartridges as described above for the preparation of samples for TLC analysis. Additionally, TLC spots of possible metabolites were scraped from the TLC plates and eluted with ethyl acetate and concentrated for analysis by GC-MS.

For analysis of the extracts a Hewlett Packard 5971 mass selective detector and 5890 series II GC with HP 7673 auto sampler tower and a 30 meter Rezteck XTI-5 column was used. The final oven temperature was maintained at 300°C. The detection limit was 1 ng or 1 μg/ml with a 1 μl injection. Mass spectrographs were compared with various libraries of mass spectrograph data prepared from known standard compounds. Several chromatograph libraries were consulted to determine the identity of metabolites of organonitrogen compounds. The presence or absence of nitrogen in various compounds was also determined by GC-AED using the nitrogen-specific wavelength of 174.2 nm for detection.

Some samples were derivatized with trimethylsilane (TMS) prior to analysis by GC-MS. The TMS-silylating reagent was prepared using 9 parts pyridine, 3 parts hexamethyldisilizane (Sigma-Aldrich catalog # 440191), and 1 part chlorotrimethylsilane (Sigma-Aldrich catalog # C72854). Approximately 25 mg of a sample (3 drops of ethyl acetate extract) was added to 2mL of TMS-silylating reagent under anaerobic conditions. The mixture was agitated/mixed and allowed to incubate at room temperature for 30-60 minutes. The samples were centrifuged and the supernatants were used for GC-MS analysis.

High Performance Liquid Chromatography

The extracts derived from growing and resting cell experiments were analyzed by HPLC. Extraction was carried out with ethyl acetate as described in the TLC section. The ethyl acetate was then evaporated completely and the residue (nonvolatile organics) was suspended in

acetonitrile before injecting into the HPLC system. A Waters system equipped with a Symmetry C_{18} (3.5 μm, 4.6 × 100 mm) column and a 600 controller was used for this purpose. Detection of compounds was carried out using a 996 photodiode array detector coupled to the HPLC system. An isocratic mobile phase of acetonitrile:water at the flowrate of 1.5ml/min was used as the running solvent.

Genetic Techniques

Methods used in genetic experiments are described in detail in the recent publications from GTI's biotechnology laboratory:

"New Host Vector System for *Thermus* spp. Based on the Malate Dehydrogenase Gene", K. J. Kayser and J. J. Kilbane II, Journal of Bacteriology 183: 1792-1795. (2001)

"Inducible and Constitutive Expression Using New Plasmid and Integrative Expression Vectors for *Thermus* sp." K. J. Kayser, J.-H. Kwak, H.-S. Park, and J. J. Kilbane II. Letters in Applied Microbiology 32: 1-7 (2001).

Electroporation of Rhodococcus erythropolis

1. Grow cells in NZ for 24-48 hours at 30^{O}C.
2. Dilute 1/5 in fresh NZ, (50 ml total volume).
3. Grow at 30^{O}C until OD_{600} of 0.6.
4. Harvest by centrifugation at 4^{O}C and wash 4 times with 1.0 ml ice cold 0.3M sucrose.
5. Resuspend cells in 1.0 ml 0.5M sucrose.
6. Add 100μl of cells to ice cold 0.2cm electroporation cuvette, and add 2 μl plasmid DNA.
7. Pulse at 25 μF, 2.4kV, 800Ω.
8. Dilute immediately in 5.0 ml NZ broth and incubate for 4 hours at 30^{O}C.
9. Concentrate cells by centrifugation and resuspend in 1.0 ml NZ.
10. Plate 100μl of cells on appropriate antibiotic plates and incubate at 30^{O}C.

RESULTS AND DISCUSSION

The work plan and milestone schedule for the project is illustrated in Figure 2. The duration of the project was continued until March 2006 as the result of a no-cost time extension. The six-month no-cost time extension was requested because of technical difficulties encountered in the isolation of an enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl. Other tasks in the project were dependent upon the availability of an appropriate enzyme so a time extension was requested and granted to allow enrichment culture experiments to proceed. A bacterial culture capable of metabolizing 2-aminobiphenyl was obtained from these enrichment culture experiments satisfying milestone 3. The genes that encode for aniline dioxygenase, that catalyzes the removal of the amine group from aniline, were cloned and are available to the project thereby satisfying milestone 4. The schedule calls for the completion of milestones 5 and 6 by the end of September 2005 and we have not yet completed those milestones. However, we are well on the way to completing these milestones and anticipate doing so within the next quarter. Detailed descriptions of the research results relevant to these milestones are contained in the text below.

FIGURE 2. WORK PLAN AND MILESTONE SCHEDULE.

Task No.	Task description	Q1 10-02 to 12-02	Q2 1-03 to 3-03	Q3 4-03 to 6-03	Q4 7-03 to 9-03	Q5 10-03 to 12-03	Q6 1-04 to 3-04	Q7 4-04 to 6-04	Q8 7-04 to 9-04	Q9 10-04 to 12-04	Q10 1-05 to 3-05	Q11 4-05 to 6-05	Q12 7-05 to 9-05	Q13 10-05 to 12-05	Q14 1-06 to 3-06
1	Enrichment culture experiments to isolate 2-aminobiphenyl degraders	X M1	X	X	X	X	X	X	X	X	X	X M3	X		
2	Directed evolution of Rhodococcus amidase gene	X	X M2	X	X	X	X	X	X	X	X	X	X		
3	Construction of pathway for C-N bond cleavage						X	X	X	X	X	X M4	X M5	X	X
4	Improving the substrate range for C-N bond cleavage											X	X M6	X M7	X M8

M1 = Multiple enrichment cultures employing inoculants from various sources will be established to obtain cultures capable of utilizing 2-aminobiphenyl as a sole nitrogen source.
M2 = The *Rhodococcus* amidase gene will be expressed in *E. coli* allowing the utilization of benzamide, toluimide, and anthranilimide as sole nitrogen sources.
M3 = A bacterial strain capable of utilizing 2-aminobiphenyl as a sole nitrogen source will be isolated.
M4 = The gene encoding an amidase/deaminase capable of selectively cleaving the C-N bond in 2-aminobiphenyl will be cloned and sequenced.
M5 = Construct an operon consisting of the carA genes from Sphingomonas sp. GTIN11 and the gene for 2-aminobiphenyl deaminase.
M6 = The substrate range for the novel C-N bond cleaving pathway will be determined.
M7 = Derivative cultures will be isolated that have improved substrate ranges for the cleavage of C-N bonds.
M8 = The ability of biocatalysts to selectively remove nitrogen from petroleum will be determined.

Selective Cleavage of Carbon-Nitrogen Bonds: Expression of CarA and AtdA in *Rhodococcus*

The development of biocatalysts with improved ability to cleave carbon-nitrogen bonds is being addressed by cloning the genes for carbazole dioxygenase and aniline dioxygenase. The removal of nitrogen from aromatic compounds like carbazole requires the cleavage of two carbon-nitrogen bonds. The cleavage of the first carbon-nitrogen bond is accomplished by the enzyme carbazole dioxygenase while an enzyme capable of selectively cleaving the second carbon-nitrogen bond has not yet been identified, but aniline dioxygenase is an appropriate candidate enzyme. The enzyme carbazole dioxygenase is encoded for by three genes: *carAa, carAc*, and *carAd*. The *carAacd* genes have been cloned and sequenced from several different microbial cultures including *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10. While an enzyme that can selectively cleave the second carbon-nitrogen bond in carbazole has not yet been identified a reasonable candidate for such an enzyme is the aniline dioxygenase from *Acinetobacter* sp. YAA. The genes that encode aniline dioxygenase (*atdA1, atdA2, atdA3, atdA4*, and *atdA5*) have been cloned. These genes will be expressed in *Rhodococcus erythropolis*, a bacterial host that tolerates exposure to petroleum. Gene expression vectors for use in *Rhodococcus erythropolis* are being constructed from plasmids pEBC26, pSRKgfp, and pRESQ.

Expression of CarA in *Rhodococcus*

The carbazole degradation pathway begins with the cleavage of the first carbon-nitrogen bond and the conversion of carbazole to 2-aminobiphenyl-2,3-diol. However, subsequent steps in this carbazole degradation pathway result in the complete destruction of carbazole and the cleavage of the second carbon-nitrogen bond is not accomplished until the molecule is nearly fully degraded and is no longer an aromatic compound. Accordingly we want to keep the first step in this pathway and replace the subsequent steps in the carbazole degradation pathway with a selective cleavage of the carbon-nitrogen bond in 2-aminobiphenyl-2,3-diol. A goal then of our research is to express the genes that encode for the first step of the carbazole degradation pathway in a host that lacks the subsequent steps in the conventional carbazole degradation pathway. The carbazole dioxygenase enzyme (CarA) that converts carbazole to 2-aminobiphenyl-2,3-diol is a multicomponent enzyme encoded by three genes; *carAacd*. The *carAacd* genes are found adjacent to and in between other carbazole degradation genes in the

carbazole degradation operons of carbazole-degrading bacteria. Therefore there are two different approaches to obtain functional *carAacd* genes in the absence of genes for subsequent steps in the carbazole degradation pathway: clone the *carAacd* genes in a host that lacks other *car* genes, and inactivate/delete the *carB* gene that encodes for the second step in the carbazole degradation pathway. In this project we have performed experiments utilizing both approaches.

The *carAacd* genes from *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10 have been cloned with their native promoters and with alternative promoters in several different vectors capable of replicating in *Rhodococcus* and *E. coli*. The vectors used include pEBC26, pSRKgfp, and pRESQ. GTI has modified these vectors to make it more convenient to insert and express genes of interest such as the *carAacd* genes. An example of a modified vector constructed in this project is pREP45boxBNsiKan.

As we attempt to construct a new metabolic pathway for the selective cleavage of C-N bonds in carbazole and as we attempt to obtain cultures that have improved levels of desulfurization activity it will be increasingly important to have gene expression vectors capable of functioning in *Rhodococcus*. One of the problems faced in attempts to express genes in *Rhodococcus* hosts is that many of the genes we are interested in do not have selectable or easily assayed phenotypes. Moreover, genes of interest may come from hosts other than *Rhodococcus* and may not have promoters that are known to function in these hosts. Accordingly, we have constructed a gene expression vector in which the kanamycin resistance gene from pSRKgfp is used as a selectable genetic marker, but the promoter of the Kan^R gene has been separated from the structural gene encoding for kanamycin resistance by the insertion of a cloning site, Nsi1. This allows the cloning of genes of interest into the Nsi1 site so that the gene is placed immediately downstream from the promoter for the Kan^R gene, and if the Kan^R gene is functionally expressed then we can be sure that our gene of interest is being transcribed. In other words, we will form a transcriptional fusion with the Kan^R gene and any gene of interest and since the same promoter will transcribe both the gene of interest and Kan^R then the selection of the culture for kanamycin resistance ensures that the gene of interest is also being expressed.

This *Rhodococcus* gene expression vector is named pREP45boxBNsiKan, and an illustration of this plasmid is shown in Figure 3. This vector, pREP45boxBNsiKan, was constructed by replacing the beta-galactosidase gene (Xba/Bgl2 fragment) of pEBC26 with the kanamycin resistance gene of pSRKgfp. However the kanamycin resistance gene was modified

using PCR to place two additional DNA sequences between the promoter (P45) and the structural gene encoding kanamycin resistance. These two DNA sequences were a boxB imperfect inverted repeat sequence and a Nsi1 site. The boxB sequence is derived from the *rrn* operon of *Rhodococcus erythropolis* IGTS8 and is thought to stabilize mRNA, most probably by providing protection from nucleases. It is hoped that the modified mRNA containing the boxB sequence will have a longer half-life in cells and will therefore result in higher levels of expression as compared to the original Kan^R gene. The Nsi1 site provides a cloning site for genes of interest allowing them to be inserted so that they are transcribed from the same promoter (P45) as the Kan^R gene.

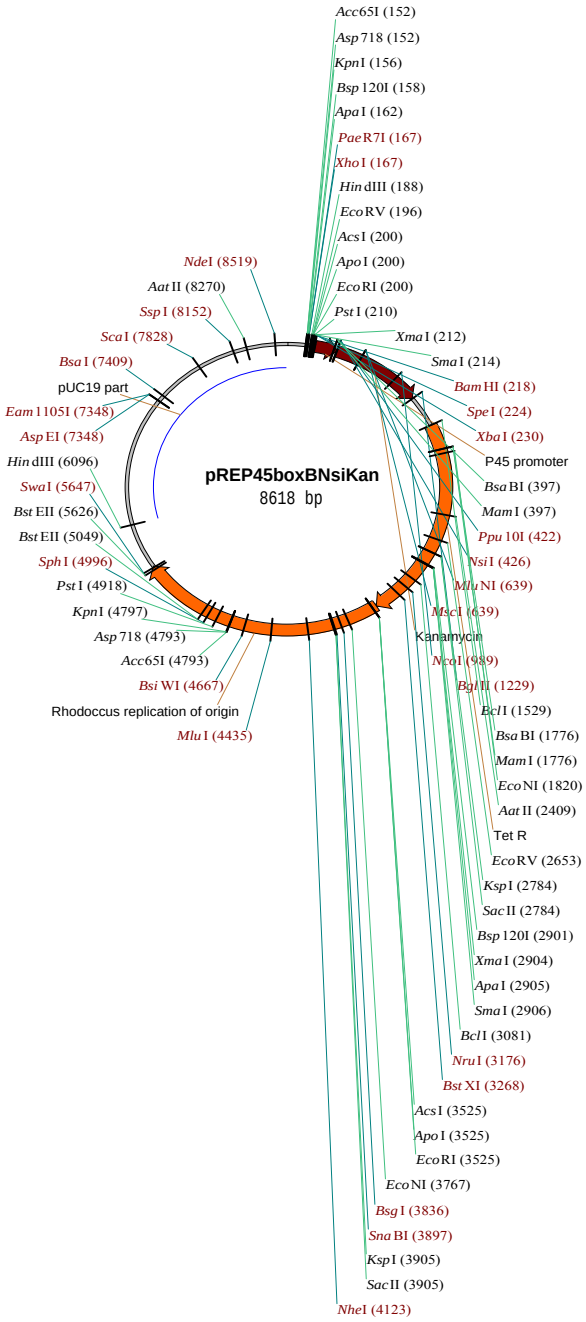


FIGURE 3. MAP OF *RHODOCOCCUS* EXPRESSION VECTOR PREP45BOXBNSIKAN.

Construction of a Derivative of *Sphingomonas* GTIN11 that Lacks CarB and CarC Enzymes

If a derivative of *Sphingomonas* sp. GTIN11 were available that had a functional CarA enzyme but lacked CarB and/or CarC enzymes it could be used to isolate the enzymes from *Pseudomonas* sp. GTIN-G4 that encode for a deaminase capable of cleaving the C-N bond in 2-aminobiphenyl. Currently *Sphingomonas* sp. GTIN11 can utilize carbazole as both a source of nitrogen and carbon, but this is because nitrogen is ultimately liberated in the overall degradation of carbazole. What we want is to create a new metabolic pathway that is specific for the cleavage of C-N bonds in carbazole. If we retain the *carA* genes that encode for the cleavage of the first C-N bond in carbazole to form 2-aminobiphenyl-2,3-diol, but delete one or more of the other genes in the carbazole degradation pathway, then the culture will convert carbazole to 2-aminobiphenyl-2,3-diol, but will not degrade it further. Accordingly, we will attempt to delete the *carB* and *carC* genes. This will be accomplished by the steps of: obtaining a DNA fragment containing the *carB* and *carC* genes as well as flanking regions, replacing the *carB* and *carC* genes with the kanamycin resistance gene from pSRKgfp, introduce this non-replicating DNA fragment into *Sphingomonas* sp. GTIN11 and select for kanamycin resistance to isolate derivatives of GTIN11 (resulting from homologous recombination at flanking DNA regions) that contain a deletion of *carB* and *carC*. Once a derivative of GTIN11 is available that lacks *carB* and *carC* we can better use this host to clone deaminase genes that may be capable of utilizing 2-aminobiphenyl-2,3-diol as a substrate.

The strategy to construct a derivative of *Sphingomonas* GTIN11 that possesses a functional CarA enzyme (encoded by *carAa, carAc*, and *carAd*), but lacks subsequent steps in the carbazole degradation pathway is pictured in Figure 4. After obtaining kanamycin-resistant derivatives as pictured in Figure 4 we have so far been unable to demonstrate the presence of a functional CarA enzyme. This may be due to a disruption of the expression of the *carAc* gene resulting from the deletion of the *carB* and *carC* genes.

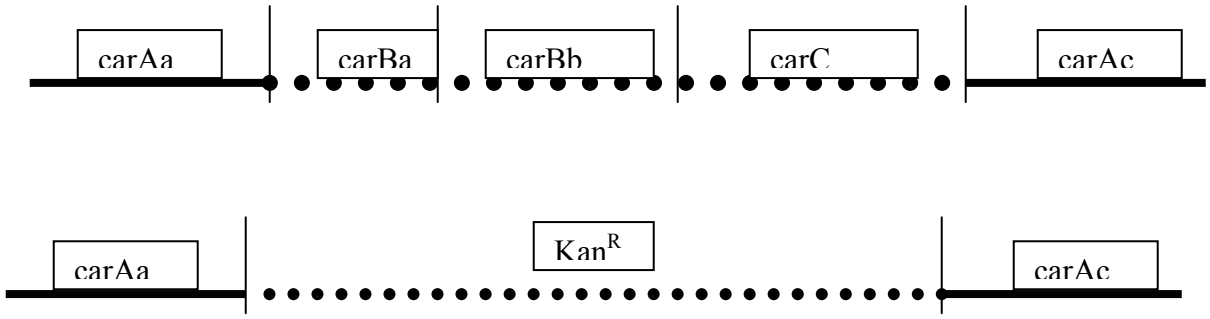
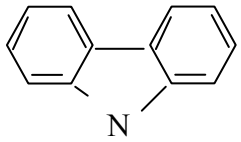


FIGURE 4. STRATEGY FOR THE REPLACEMENT OF THE *CARBA*, *CARBB*, AND *CARC* GENES OF SPHINGOMONAS GTIN11 WITH A GENE ENCODING KANAMYCIN RESISTANCE.

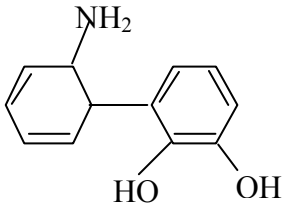
Isolation of 2ABP Degrading Bacterial Culture

The selective removal of nitrogen from petroleum can complement hydrotreatment for upgrading petroleum and create petroleum products that have better stability. It is important to note that recent investigations reported in the literature [3] show that the typical biochemical pathways for the metabolism of carbazole are not suitable for upgrading petroleum. 2ABP is converted to 2-aminobiphenyl-2,3-diol by the first step in previously characterized carbazole degradation pathways. It has been speculated that the cleavage of one of the two C-N bonds in carbazole, and related compounds, could be sufficient to allow upgrading of petroleum in combination with hydrotreating. However, the study by Bressler et al. [2, 3] demonstrated that compounds such as 2-aminobiphenyl-2,3-diol are transformed by the hydrotreatment process resulting in ring closure, condensation, and polymerization. Deamination was not observed. The implication of this for the current project is that biochemical cleavage of both C-N bonds in carbazole is required for upgrading petroleum and previously characterized biochemical pathways for carbazole biodegradation are not appropriate. The newly isolated 2ABP-degrading culture *Pseudomonas* sp. GTIN-G4 may contain an enzyme capable of cleaving the C-N bond in 2-aminobiphenyl-2,3-diol that could be used to help construct the first biochemical pathway for the selective removal of nitrogen from carbazole. Accordingly, the metabolites of 2ABP, and related compounds, formed by *Pseudomonas* sp. GTIN-G4 were investigated.

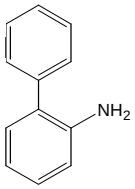
FIGURE 5. STRUCTURE OF ORGANONITROGEN COMPOUNDS USED IN GROWTH TESTS WITH PSEUDOMONAS SP. GTIN-G4



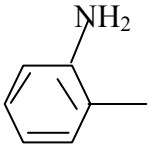
Carbazole



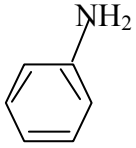
2-aminobiphenyl-2,3-diol



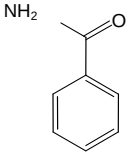
2-aminobiphenyl



ortho-toluidine



Aniline



Benzamide

Pseudomonas sp. GTIN-G4 was isolated based on its ability to grow in a defined mineral salts medium containing 2-aminibiphenyl (2ABP) as the sole nitrogen source. Initial microbial growth tests utilized a stock solution of 100 millimolar 2ABP dissolved in ethanol. Growth experiments employed a final concentration of 1 mM 2ABP in MMN medium incubated aerobically at 30^{o}C. *Pseudomonas* sp. GTIN-G4 grew under these conditions and appeared to be utilizing 2ABP as a sole source of nitrogen and carbon. After repeated testing of the culture it became clear that ethanol was a good carbon source for the growth of the culture. Subsequent growth tests exclusively used crystals or neet liquids of test compounds rather than stock solutions dissolved in ethanol.

The results of a typical growth test are shown in Table 2 in which the ability of organic and inorganic nitrogen sources were tested to see if they support the aerobic growth of *Pseudomonas* sp. GTIN-G4. The results appear to indicate that carbazole, 2ABP, o-toluidine, and diphenylamine can be used as nitrogen sources but not as carbon sources. However, unexpected growth was also observed when ethanol was used as a carbon source, but no nitrogen source was provided. *Pseudomonas* sp. GTIN-G4 is a fragile culture that frequently fails to yield growth upon subculturing so that growth test must be repeated multiple times before it can be concluded with confidence that a given condition will allow growth of this culture. This is why it was not initially observed that *Pseudomonas* sp. GTIN-G4 could grow in the absence of a specifically supplied organic or inorganic nitrogen compound. However, repeated testing yielded the results summarized in Table 2. The test substrates 2ABP, o-toluidine, and diphenylamine were transformed to colored products in growth experiments and these colored products were not observed in sterile controls. It was clear then that *Pseudomonas* sp. GTIN-G4 was metabolizing 2ABP, o-toluidine, and diphenylamine but it was not clear if these compounds were serving as nitrogen sources for the growth of the culture.

Table 2. Aerobic Growth of *Pseudomonas* sp. GTIN-G4 with Various Nitrogen Sources

Carbon Source	**Nitrogen Source**	**Growth**	**Color of Sample**
None	None	-	clear
ethanol	None	++	gray
ethanol	NH_4Cl	++	gray
ethanol	NH_4NO_3	++	gray
ethanol	carbazole	++	gray
ethanol	2-aminobiphenyl	++	copper/brown
ethanol	o-toluidine	++	light brown
ethanol	diphenylamine	++	slightly purple
None	NH_4NO_3	-	clear
None	carbazole	-	clear
None	2-aminobiphenyl	-	clear

The growth of the culture in the apparent absence of a nitrogen source suggested the possibility that the culture could fix atmospheric nitrogen. To investigate this possibility, growth tests were performed in sealed serum bottles using pure oxygen or air as the headspace gas. Multiple growth experiments were performed and the results are summarized in Table 3, which illustrated that no growth was obtained when pure oxygen was used while air did yield growth supporting the conclusion that *Pseudomonas* sp. GTIN-G4 can fix N_2 gas. However, the failure of the culture to grow in the presence of pure oxygen even when NH_4Cl or NH_4NO_3 were provided raised the possibility that perhaps pure oxygen was toxic to the culture.

Table 3. Growth of *Pseudomonas* sp. GTIN-G4 in the Absence of Nitrogen Gas

Carbon Source	**Nitrogen Source**	**Headspace Gas**	**Growth**
None	None	O_2	-
ethanol	None	O_2	-
ethanol	NH_4Cl	O_2	-
ethanol	NH_4NO_3	O_2	-
ethanol	2-aminobiphenyl	O_2	-
ethanol	o-toluidine	O_2	-
ethanol	diphenylamine	O_2	-
None	2-aminobiphenyl	O_2	-
None	None	Air	-
ethanol	None	Air	++
ethanol	2-aminobiphenyl	Air	++

This possibility was investigated in experiments summarized in Table 4 in which the growth of *E. coli* and *Pseudomonas* sp. GTIN-G4 were compared using various concentrations of oxygen. To achieve the desired concentration of oxygen the sealed serum bottles were first sparged for 2 minutes with pure oxygen then the required volume of gas was removed and replaced with helium. However, growth of *Pseudomonas* sp. GTIN-G4 was not obtained even with 25% oxygen, which is very near the oxygen concentration in air. Therefore some experiments were performed in which the sealed serum bottles were first sparged with helium and then the required volume of gas was removed and replaced by oxygen in order to achieve the

desired oxygen concentration. As shown in Table 4, *Pseudomonas* sp. GTIN-G4 is sensitive to exposure to pure oxygen while *E. coli* is not.

Table 4. Growth of *Pseudomonas* sp. GTIN-G4 and *Escherichia coli* with Various Concentrations of Oxygen.

Bacterial Culture	**Media composition**	**Headspace Gas**	**Growth**
GTIN-G4	MMN,ethanol, NH_4NO_3	100% O_2	-
GTIN-G4	MMN,ethanol, NH_4NO_3	50% O_2	-
GTIN-G4	MMN,ethanol, NH_4NO_3	25% O_2	-
GTIN-G4	MMN,ethanol, NH_4NO_3	20% O_2, He first	+
E. coli	L Broth	100% O_2	++
E. coli	L Broth	50% O_2	++
E. coli	L Broth	25% O_2	++
E. coli	L Broth	20% O_2, He first	++

To more fully define the growth capabilities of *Pseudomonas* sp. GTIN-G4 experiments were performed using cultures initially sparged with helium and subsequently adjusted so the gas composition was 20% oxygen. The results of such tests are shown in Table 5 where it is observed that when growth of *Pseudomonas* sp. GTIN-G4 occurs in the absence of N_2 gas colored metabolites of 2ABP, o-toluidine, or diphenylamine are not observed. Growth of *Pseudomonas* sp. GTIN-G4 with air is required for the production of colored products derived from organonitrogen substrates.

Table 5. Growth of *Pseudomonas* sp. GTIN-G4 with Various Organonitrogen Compounds in the Absence of nitrogen Gas

Nitrogen Source	**Headspace Gas**	**Growth**
NH_4Cl	20% O_2/80% He	++, clear
NH_4NO_3	20% O_2/80% He	++, clear
NH_4NO_3, 2-aminobiphenyl	20% O_2/80% He	++, clear
NH_4NO_3, o-toluidine	20% O_2/80% He	++, clear
NH_4NO_3, diphenylamine	20% O_2/80% He	++, clear
2-aminobiphenyl	20% O_2/80% He	-, clear
o-toluidine	20% O_2/80% He	-, clear
diphenylamine	20% O_2/80% He	-, clear
2-aminobiphenyl	Air	++, copper/brown
o-toluidine	Air	++, light brown
diphenylamine	Air	++, slightly purple

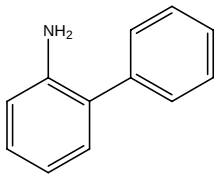
Pseudomonas sp. GTIN-G4 metabolizes 2-aminobiphenyl, diphenylamine, o-toluidine, and aniline in liquid cultures to produce colored metabolites that are not observed in sterile controls or with cultures of other bacteria incubated with these substrates. GC-MS analyses identified some unique products that appear to result from the metabolism of *Pseudomonas* sp. GTIN-G4. Mass spectral analysis was performed on aqueous extract samples in an attempt to identify potential biodegradation byproducts formed by *Pseudomonas* sp. GTIN-G4 from 2-aminobiphenyl, diphenylamine, and o-toluidine. Compounds were found in all three sample types suggesting a substitution of a hydrogen on the nitrogen atom with a CHO group. The proposed structure was confirmed for the diphenylamine by comparing it to a standard of N,N-diphenylformamide. The structures related to the 2-aminobiphenyl and o-toluidine samples are

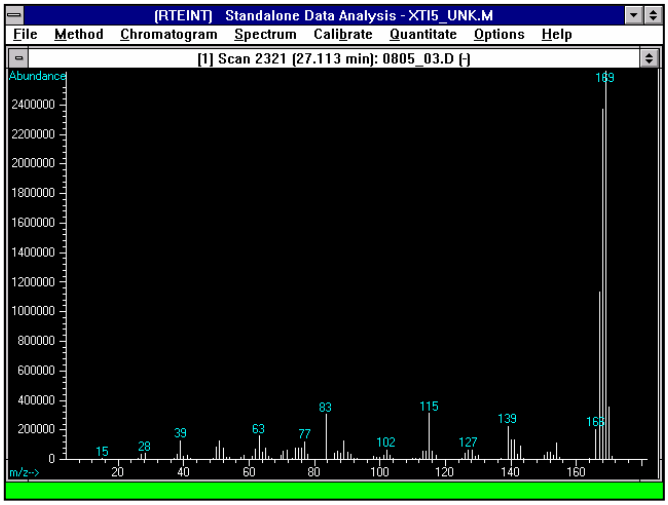
based on the similarities of their mass spectra to that of N,N-diphenylformamide. GC-MS data illustrating the formaldehyde-modified derivatives of 2ABP, o-toluidine, and diphenylamine produced by *Pseudomonas* sp. GTIN-G4 are shown in Figures 6, and 7 respectively. Similar results were also obtained for the metabolism of aniline by *Pseudomonas* sp. GTIN-G4 (data not shown).

The microbial modification of chemical compounds by the addition of a formaldehyde group is without precedent. The addition of methyl groups to detoxify organic and metal compounds is a well-known capability of many microorganisms and fungi [1]. Moreover, the addition of a methyl group to a nitrogen atom in place of a hydrogen atom, such as the biochemical conversion of pyridine to N-methylpyridine, is well known. Similarly, the addition of carboxyl groups to organic compounds by aerobic [4] and anaerobic microorganisms [28], and the addition of carbonyl groups [6] are described in the literature. However, there are no reports of microbial cultures that are capable of modifying organic compounds by the addition of formaldehyde groups except in the degradation of formaldehyde where formaldehyde reacts with ribulose-5-phosphate to form D-arabino-3-hexulose-6-phosphate, which is subsequently isomerized to fructose-6-phosphate [22]. The formation of N-formamide derivatives of 2-aminobiphenyl, diphenylamine, and o-toluidine is a unique capability of *Pseudomonas* sp. GTIN-G4, but it is not clear if this will facilitate the eventual removal of nitrogen from these molecules. Since *Pseudomonas* sp. GTIN-G4 is capable of fixing nitrogen it is difficult to determine if organically bound nitrogen is liberated in microbiological experiments that include this culture. Accordingly it does not appear to be worthwhile to continue an investigation of this culture, as it does not clearly aid in the removal of nitrogen from compounds typically present in petroleum.

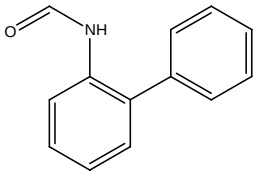
FIGURE 6. MASS SPECTRA OF 2-AMINOBIPHENYL AND A METABOLITE PRODUCED BY *PSEUDOMONAS* SP. GTIN-G4.

2-aminobiphenyl





2-formamidylbiphenyl



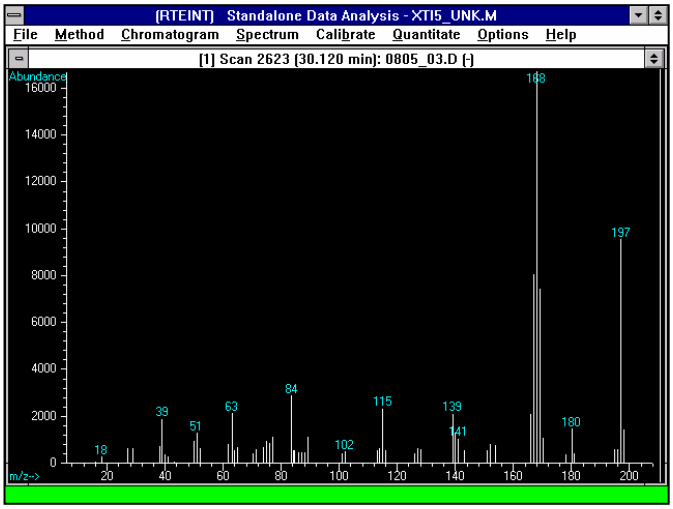
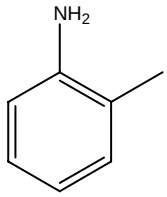
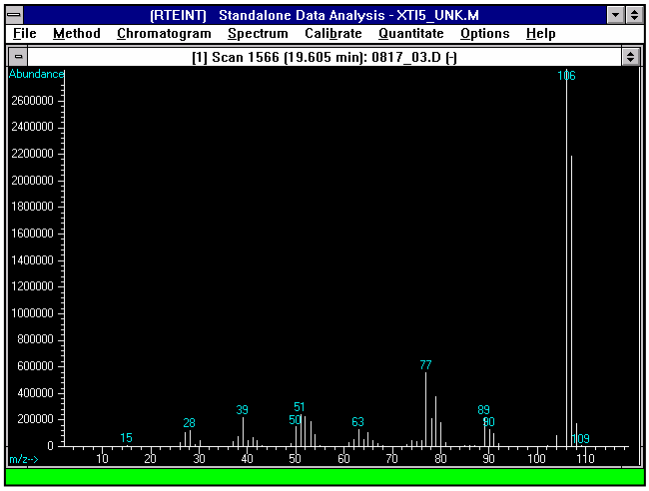


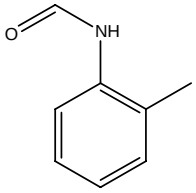
FIGURE 7. MASS SPECTRA OF O-TOLUIDINE AND DIPHENYLAMINE AND THE CORRESPONDING METABOLITES PRODUCED BY *PSEUDOMONAS* SP. GTIN-G4.

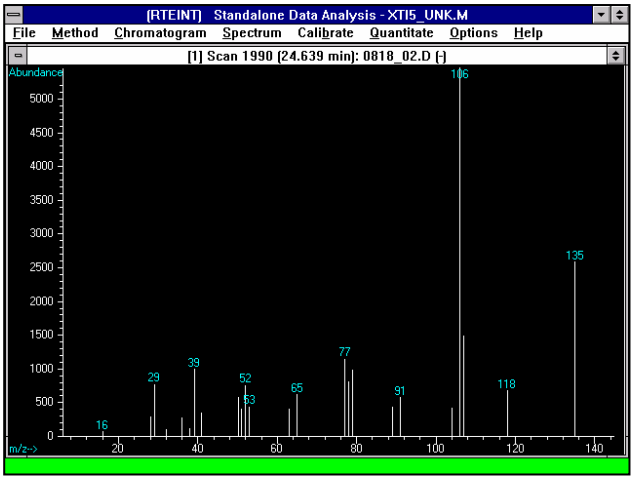
o-toluidine



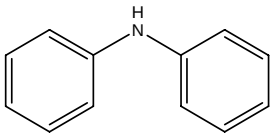


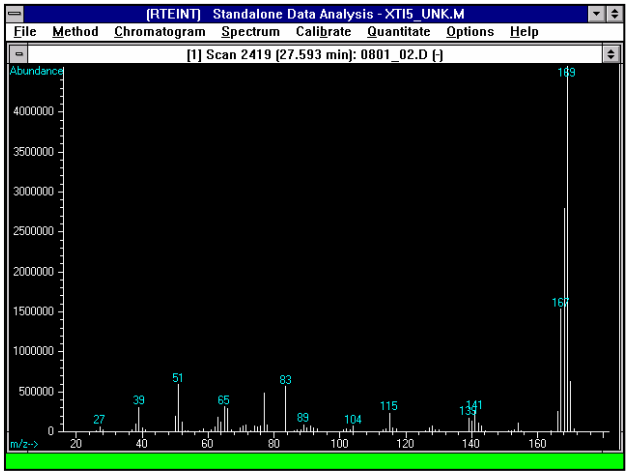
2-methylbenzylformamide



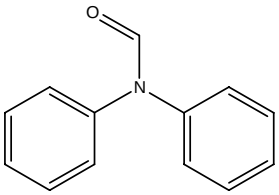


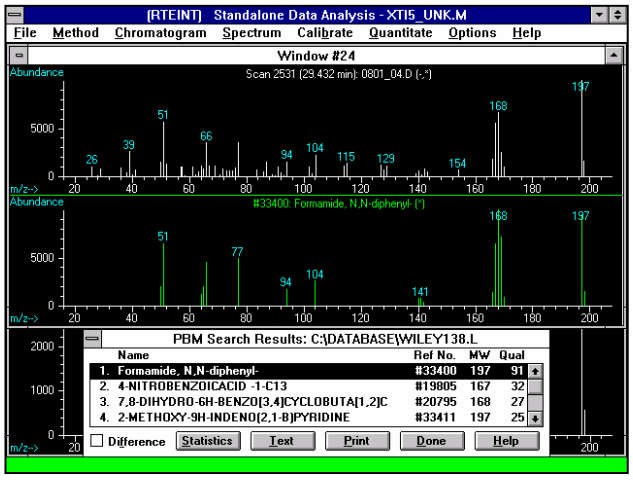
Diphenylamine





N,N-diphenyl formamide





Cloning and Expression of the *atdA1-5* Genes for Aniline Dioxygenase

Since an enzyme capable of directly cleaving the carbon-nitrogen bond in 2-aminobiphenyl has not yet been isolated then the best alternative is aniline dioxygenase. Aniline dioxygenase, AtdA, is a multicomponent enzyme isolated from *Acinetobacter* sp. Strain YAA involved in the simultaneous deamination and oxygenation of aniline [10] and o-toluidine, see Figure 8. The genes encoding for AtdA was found to have 5 open reading frames (ORFs), atdA1-A5.

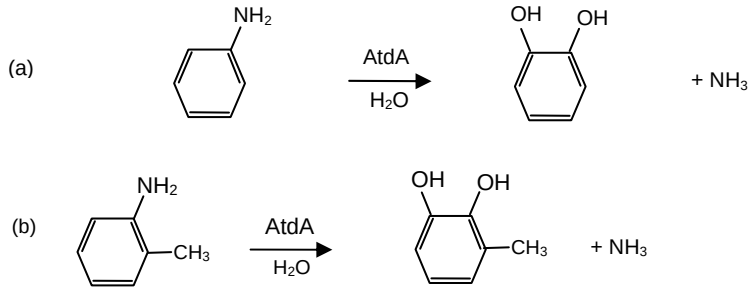


FIGURE 8. DEAMINATION AND OXYGENATION OF (A) ANILINE AND (B) O-TOLUIDINE BY ATDA.

The plasmid pAS93, consisting of the five *atdA* open reading frames (ORFs) cloned into a pUC19 vector, [10] was obtained from Dr Takeo in the Department of Applied Chemistry, Himeji Institute of Technology, Japan. *E. coli* JM109 was used as host for the pAS93 plasmid.

The *atdA1-5* genes will be cloned and expressed in *Rhodococcus* hosts using the same vectors described above for expression of *carAacd* genes. By simultaneously expressing the *carAacd* and the *atdA1-5* genes in the same host it is hoped that natural selection for growth with carbazole as a sole source of nitrogen will result in the creation of a new metabolic pathway for the selective cleavage of both carbon-nitrogen bonds in carbazole.

CONCLUSIONS AND FUTURE EXPERIMENTS

Enrichment culture experiments designed to isolate a culture capable of cleaving the carbon-nitrogen bond in 2-aminobiphenyl, and thus being capable of providing an enzyme for the cleavage of the second carbon-nitrogen bond in carbazole, resulted in the isolation of a unique microbial culture *Pseudomonas* sp. GTIN-G4. *Pseudomonas* sp. GTIN-G4 is capable of metabolizing 2-aminobiphenyl and related compounds, but does not appear to be capable of cleaving the carbon-nitrogen bond. Instead it has the unprecedented ability to modify organonitrogen compounds by replacing a hydrogen bound to the nitrogen atom with a formaldehyde group. This is a very interesting metabolic ability, but it does not allow the removal of nitrogen from organonitrogen compounds so this culture will not be examined further. The time remaining in this project does not allow continued enrichment culture experiments to isolate a culture that contains an enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl. It is not known how long such an effort might take and if it can be successful within the time remaining in the project. Therefore we will focus on the use of enzymes/genes that are available to the project and known to selectively cleave C-N bonds in carbazole or molecules chemically related to carbazole.

Future experiments will focus on using molecular genetics to obtain derivatives of *Rhodococcus erythropolis* IGTS8 that are more efficient biocatalysts for the removal of nitrogen from petroleum. Specifically, the *carAacd* genes from *Sphingomonas* sp. GTIN11 and/or *Pseudomonas resinovorans* CA10 will be expressed simultaneously with the *atdA1-5* genes from *Acinetobacter* sp. YAA in a *Rhodococcus* host. The simultaneous expression of these two sets of genes in the same host may allow for the creation of a novel biochemical pathway for the selective cleavage of both C-N bonds in carbazole.

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