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

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ABSTRACT

The objective of the project is to develop biochemical pathways for the selective cleavage of C-N bonds in molecules found in petroleum. The initial phase of the project was focused on the isolation or development of an enzyme capable of cleaving the C-N bond in aromatic amides, specifically 2-aminobiphenyl. The objective of the second phase of the research will be to construct a biochemical pathway for the selective removal of nitrogen from carbazole by combining the *carA* genes from *Sphingomonas sp*. GTIN11 with the gene(s) encoding an appropriate deaminase. The objective of the final phase of the project will be to develop derivative C-N bond cleaving enzymes that have broader substrate ranges and to demonstrate the use of such strains to selectively remove nitrogen from petroleum.

During the first year of the project (October, 2002-September, 2003) enrichment culture experiments resulted in the isolation of microbial cultures that utilize aromatic amides as sole nitrogen sources, several amidase genes were cloned and were included in directed evolution experiments to obtain derivatives that can cleave C-N bonds in aromatic amides, and the *carA* genes from *Sphingomonas sp*. GTIN11, and *Pseudomonas resinovorans* CA10 were cloned in vectors capable of replicating in *Escherichia coli.* During the second year of the project (October, 2003-September, 2004) enrichment culture experiments succeeded in isolating a mixed bacterial culture that can utilize 2-aminobiphenyl as a sole nitrogen source, directed evolution experiments were focused on the aniline dioxygenase enzyme that is capable of deaminating aniline, and expression vectors were constructed to enable the expression of genes encoding C-N bond cleaving enzymes in *Rhodococcus* hosts. The construction of a new metabolic pathway to selectively remove nitrogen from carbazole and other molecules typically found in petroleum should lead to the development of a process to improve oil refinery efficiency by reducing the poisoning, by nitrogen, of catalysts used in the hydrotreating and catalytic cracking of petroleum. Aromatic compounds such as carbazole are representative of the difficult-to-treat organonitrogen compounds most commonly encountered in petroleum. There are two C-N bonds in carbazole and the construction of a metabolic pathway for the removal of nitrogen from carbazole will require enzymes capable cleaving both C-N bonds. A multi-component enzyme, carbazole dioxygenase, which can selectively cleave the first C-N bond has been identified and the genes that encode this enzyme have been cloned, sequenced, and are being expressed in *Rhodococcus erythropolis*, a

bacterial culture that tolerates exposure to petroleum. An enzyme capable of selectively cleaving the second C-N bond in carbazole has not yet been identified, but enrichment culture experiments have recently succeeded in isolating a bacterial culture that is a likely candidate and may possess a suitable enzyme. Research in the near future will verify if a suitable enzyme for the cleavage of the second C-N bond in carbazole has indeed been found, then the genes encoding a suitable enzyme will be identified, cloned, and sequenced. Ultimately genes encoding enzymes for selective cleavage of both C-N bonds in carbazole will be assembled into a new metabolic pathway and the ability of the resulting bacterial culture to remove nitrogen from petroleum will be determined.

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EXECUTIVE SUMMARY

The objective of the project is to develop biochemical pathways for the selective cleavage of C-N bonds in molecules found in petroleum. The initial phase of the project was focused on the isolation or development of an enzyme capable of cleaving the C-N bond in aromatic amides, specifically 2-aminobiphenyl. The objective of the second phase of the research will be to construct a biochemical pathway for the selective removal of nitrogen from carbazole by combining the *carA* genes from *Sphingomonas sp*. GTIN11 with the gene(s) encoding an appropriate deaminase. The objective of the final phase of the project will be to develop derivative C-N bond cleaving enzymes that have broader substrate ranges and to demonstrate the use of such strains to selectively remove nitrogen from petroleum.

Some difficulties were encountered that resulted in the revision of project milestones, with the concurrence of the DOE Project Manager. Specifically, while a mixed culture capable of utilizing 2-aminobiphenyl has been obtained a pure culture that possesses this ability has not yet been isolated. The lack of a pure culture that can selectively cleave the C-N bond in 2-aminobiphenyl has prevented us from cloning and sequencing a gene encoding this trait, and using such a gene to construct a novel metabolic pathway for the selective cleavage of both C-N bonds in carbazole. There are two C-N bonds in carbazole and the construction of a metabolic pathway for the removal of nitrogen from carbazole will require enzymes capable cleaving both C-N bonds. A multi-component enzyme, carbazole dioxygenase, which can selectively cleave the first C-N bond has been identified and the genes that encode this enzyme have been cloned, sequenced, and are being expressed in *Rhodococcus erythropolis*, a bacterial culture that tolerates exposure to petroleum. An enzyme capable of selectively cleaving the second C-N bond in carbazole has not yet been identified, but enrichment culture experiments have recently succeeded in isolating a bacterial culture that is a likely candidate and may possess a suitable enzyme. Research in the near future will verify if a suitable enzyme for the cleavage of the second C-N bond in carbazole has indeed been found, then the genes encoding a suitable enzyme will be identified, cloned, and sequenced. Ultimately genes encoding enzymes for selective cleavage of both C-N bonds in carbazole will be assembled into a new metabolic pathway and the ability of the resulting bacterial culture to remove nitrogen from petroleum will be determined.

The genes for the carbazole degradation operon of *Sphingomonas* sp. GTIN11 have been cloned and sequenced. While the carbazole degradation pathway encoded by *Sphingomonas* sp. GTIN11 appears to be biochemically the same as some previously characterized carbazole degrading cultures such as *Pseudomonas resinovorans* CA10, the arrangement and DNA sequence of the carbazole degradation genes in *Sphingomonas* sp. GTIN11 is unique. Specifically, the carbazole degrading enzymes encoded by *Sphingomonas* sp. GTIN11 are 60% or less similar to previously characterized carbazole-degrading enzymes and the degree of similarity is even less at the level of DNA sequences. The carbazole dioxygenase enzyme is encoded by three genes: *carA, carAc*, and *carAd*. These *carA* genes have been cloned from both *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10 and expressed in *Escherichia coli.* These *carA* genes have also been introduced into *Rhodococcus erythropolis*, but functional expression has not yet been obtained. A focus of recent research has been the development of gene expression vectors for *Rhodococcus erythropolis*. The ability to efficiently express genes in *Rhodococcus erythropolis* will assist in the development of effective biocatalysts for the treatment of petroleum because this species tolerates exposure to petroleum and some strains posses the desulfurization pathway for the selective cleavage of C-S bonds. An ultimate goal of this project is to construct a novel metabolic pathway for the selective cleavage of C-N bonds in substrates such as carbazole. This will be accomplished by combining the *carA* genes with genes that encode an enzyme capable of selectively cleaving the second C-N bond in carbazole. Accordingly, enrichment culture experiments and directed evolution experiments designed to obtain enzymes capable of selectively cleaving the second C-N bond in carbazole, typified by the substrate 2-aminobiphenyl, are a key project activity.

Obtaining a deaminase capable of cleaving C-N bonds in aromatic compounds, particularly 2-aminobiphenyl, is essential to the creation of a metabolic pathway for the selective removal of nitrogen from carbazole and related organonitrogen compounds typically found in petroleum. A mixed culture, SL1-5, capable of growing with 2-aminobiphenyl as the sole nitrogen source was isolated. This mixed culture has been demonstrated to utilize 2-aminobiphenyl as a sole nitrogen source in at least six independent experiments and all of the controls performed as expected. This is a major project milestone as the isolation of an enzyme that can cleave the second carbon-nitrogen bond in carbazole (such as the model compound 2-aminobiphenyl) has been a goal of this project since its inception, and this is the first culture that

appears to metabolize 2-aminobiphenyl efficiently. This mixed culture must now be processed to obtain a pure culture that possesses the ability to utilize 2-aminobiphenyl and to characterize the substrate range of the culture. This will be the focus of research in the near future. Other project activities included performing DNA transformations of bacterial strains *Rhodococcus erythropolis, Sphingomonas* sp GTIN11, and *Pseudomonas ayucida* IGTN9. Cloning vectors are already available for use in *Rhodococcus* so *carA, qorMLS*, and *triA* genes were transformed into *Rhodococcus*. *Sphingomonas* sp GTIN11, and *Pseudomonas ayucida* IGTN9 have never been used as hosts in genetic experiments so various vectors were transformed into these cultures to determine which replicons, drug resistance genes, and promoters will function in these hosts. In future experiments we plan to inactivate the *carB* genes in *Sphingomonas* sp GTIN11 and to introduce various deaminase genes into *Sphingomonas* sp GTIN11, and *Pseudomonas ayucida* IGTN9.

Another recent accomplishment was the demonstration that the Gibbs assay can be used to investigate the substrate range of C-N bond cleaving enzymes that act on aromatic substrates, and can be used to monitor the activity of such enzymes. The Gibbs reagent reacts with aromatic hydroxyl groups to yield colored products that can be detected and quantified spectrophotometrically. The Gibbs assay proved to be useful in monitoring the activity of C-S bond cleaving enzymes that act on substrates such as dibenzothiophene, but during this past year we demonstrated that the Gibbs assay could be used to investigate the activity of enzymes relevant to the metabolism of organonitrogen compounds.

Research in the near future will include characterization of the 2-aminobiphenyl-degrading culture SL1-5, and continuing experiments to express heterologous genes relevant to C-N bond cleavage in *Rhodococcus, Sphingomonas, Gordonia,* and *Pseudomonas* hosts.

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 in petroleum can foul catalysts decreasing the efficiency of hydrotreating and catalytic cracking processes. 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 of 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[2, 9, 25]. 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. 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[4, 8, 19, 25]. 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[10].

There is currently no biochemical pathway, or thermochemical process, for the selective removal of nitrogen from compounds typically present in petroleum[4, 25]. 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[12]. 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[2, 9, 15]. 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[1, 7, 12-14, 16, 18, 20, 23, 24]. A microorganism capable of selectively cleaving C-N bonds in quinoline and removing nitrogen from petroleum was isolated and characterized[11] (by GTI) but no genetic information is available concerning this culture. And no other cultures capable of selectively cleaving C-N bonds in molecules relevant to petroleum have been characterized biochemically and genetically. 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[1, 7, 12-14, 17, 21-24]. 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 hetorocyclic 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.

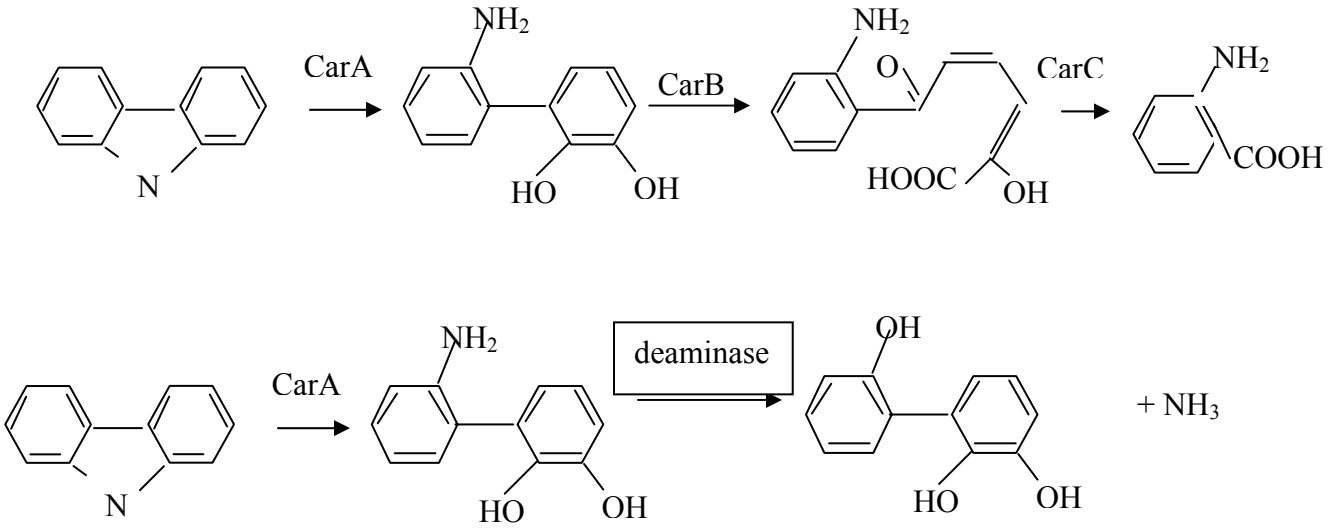


FIGURE 1. CARBAZOLE DEGRADATION PATHWAYS.

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 [12]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[3, 5]. This project will use enrichment culture, and directed evolution to isolate and/or create an amidase that will recognize 2'-aminobiphenyl-2,3-diol as a substrate. The gene encoding an appropriate amidase 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[7, 20, 23] so that complete biodegradation of carbazole would be avoided and the final product would be 2',2,3-trihydroxybiphenyl (or a similar compound). Subsequent tests of petroleum biotreatment will also be performed.

A two-fold approach will be employed to obtain an appropriate deaminase: enrichment cultures, and directed evolution. 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 initial experiments will employ 2-aminobiphenyl. The best candidate deaminases for use in directed evolution have limited ability to metabolize aromatic amides and their substrate range includes benzamide, toluamide, and anthranilamide[3, 5]. The amidase gene from *Rhodococcus* sp. MP50 (GenBank X54074) will be used as the target of directed evolution experiments because of its substrate range and the availability of gene expression vectors for *Rhodococcus*. The genetic work can be performed in *E. coli* and mutant candidates can be selected for by growth using 2-aminobiphenyl as a sole nitrogen source. Appropriate mutants will have a selective advantage facilitating their isolation. Once bacterial cultures are available that can deaminate 2-aminobiphenyl, then the amidase 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. Then additional directed evolution and enrichment culture experiments will be performed to obtain derivatives with expanded substrate ranges so that C-N bond cleavage in a greater variety of compounds relevant to petroleum can be accomplished.

This project is relevant to DOE's mission both because of its objective and its approach. 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. The objective of the project is to develop biochemical pathways for the selective cleavage of C-N bonds in molecules found in petroleum. The approach of employing metabolic engineering and directed evolution will demonstrate methodology to create

biochemical pathways that have the requisite selectivity, substrate range and specific activity for industrial applications. Biotechnology may one day solve many problems confronting the petroleum industry today, but a biorefining process will have to operate on a far greater scale and at less cost than any current biotechnology process. For any process to be viable in the petroleum industry it must be capable of treating the complex mixture of chemicals that comprise petroleum, and be able to treat huge volumes in a cost effective manner. Many enzymes catalyze reactions relevant to DOE interests but they must be improved in numerous ways before practical, economical bioprocesses can be developed. The metabolic engineering and directed evolution approaches demonstrated in this project will be widely applicable for the development of bioprocesses relevant to the energy industry.

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 U.S. 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[19].

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[26]. Energy statistics indicate that the U.S. imports 65% of its oil demand[10]. New technologies, such as the proposed work, that will allow a greater utilization of heavy oils and residuum while still maintaining refinery efficiency and environmental protection, will contribute to national security by decreasing dependence on foreign oil. 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[10, 19]. 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.

A three-year research program is needed for the development of a biological process for the removal of nitrogen from petroleum. A work plan consisting of four tasks, shown in Figure

2, will be followed to accomplish the objective of this project: 1) Enrichment Culture Experiments to Isolate 2-aminobiphenyl Degraders, 2) Directed Evolution of *Rhodococcus* Amidase Gene, 3) Construction of Pathway for C-N Bond Cleavage, and 4) Improving the Substrate Range for C-N Bond Cleavage. The initial steps in metabolism of carbazole by *Sphingomonas sp.* GTIN11 accomplishes the selective cleavage of one of the two C-N bonds in carbazole and tasks 1 and 2 represent two different strategies to obtain an enzyme capable of selectively cleaving the second C-N bond. Task three will construct a pathway for the selective cleavage of both C-N bonds in carbazole by combining the *carA* genes from *Sphingomonas sp.* GTIN11 with an appropriate amidase gene developed in task 1 and/or 2. Finally, task 4 will obtain derivatives of the C-N bond cleavage pathway that will accept a broader range of substrates, and will demonstrate the effectiveness of these improved biocatalysts to selectively remove nitrogen from petroleum.

The task and milestone schedule shown in Figure 2 reflects modifications made in September, 2004 in consultation with the DOE project manager.

FIGURE 2. TASK SCHEDULE AND MILESTONE CHART

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
1	Enrichment culture experiments to isolate 2-aminobiphenyl degraders	X M1	X	X	X	X	X						
2	Directed evolution of *Rhodococcus* amidase gene	X	X M2	X	X	X	X M3	X	X	X			
3	Construction of pathway for C-N bond cleavage						X	X	X	X M4	X	X M5	
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 capable of selectively cleaving the C-N bond in 2-aminobiphenyl will be cloned and sequenced.

M5 = An operon will be constructed consisting of the *carA* genes from *Sphingomonas* sp. GTIN11 and the gene for 2-aminobiphenyl amidase.

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.

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 3-20 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 3 -20mM. 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.

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, 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 1 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. In this 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 in 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 controls for conditions 2 and 3.

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.

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 ice cold 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.

Plate 100μl of cells on appropriate antibiotic plates and incubate at 30^{O}C.

RESULTS AND DISCUSSION

Enrichment Culture Experiments

Enrichment culture experiments were performed using soil from a variety of petroleum/hydrocarbon-contaminated sites. Of primary interest is the isolation of a culture capable of utilizing 2'aminobiphenyl-2,3-diol, which is the product of the conversion of carbazole by the CarA enzyme, carbazole 1,9a-dioxygenase. Initially the nitrogen-free recipe ModA and then the improved recipe MMN described in the Materials and Methods section was used. The organonitrogen chemicals used in enrichment culture experiments included 2-aminobiphenyl, benzamide, aniline, 4,4'-azodianiline, ortho-, meta-, and para-toluidine, quinoline, pyridine, carbazole, quinazoline, piperidine, pyrolidine, and triazine. A mixture of glucose, glycerol, and succinate was employed as a carbon source to encourage the growth of a wide range of microbial species.

Enrichment cultures were successful in obtaining several mixed cultures that were capable of utilizing organonitrogen compounds as sole nitrogen sources and these mixed cultures were then processed to obtain pure bacterial cultures capable of utilizing organonitrogen compounds as sole nitrogen sources. The potentially useful cultures that were isolated included *Rhodococcus globerulus, Sphingomonas yanoikuyae, Methylobacterium radiotolerans, Gordonia bronchialis,* and *Rhodopseudomonas palustris* that were capable of metabolizing carbazole,aniline, or 4,4'-azodianiline. Of all of the pure microbial cultures listed above, none proved to be worth detailed characterization because no unique enzyme capable of selectively cleaving C-N bonds could be demonstrated. Upon further characterization it was found that these cultures did not selectively cleave C-N bonds in organonitrogen compounds, but would also utilize organonitrogen compounds as sole carbon sources. Therefore none of these cultures offered advantages versus *Sphingomonas* sp. GTIN11 that is a carbazole-degrading microorganism that was previously characterized.

The carbazole degradation operon of *Sphingomonas* sp. GTIN11 has been cloned and sequenced and contains genes that specify the CarA enzyme (the *carA, carAc*, and *carAd* genes) that is responsible for the cleavage of the first C-N bond in carbazole such that carbazole is converted to 2-aminobiphenyl-2,3-diol. However, an enzyme that selectively cleaves the C-N bond in 2-aminobiphenyl-2,3-diol is lacking. Therefore, obtaining such an enzyme that selectively cleaves the C-N bond in 2-aminobiphenyl-2,3-diol became the key focus of

enrichment culture experiments. Once an appropriate deaminase enzyme is found that can selectively cleave the C-N bond in 2-aminobiphenyl-2,3-diol the gene(s) that encode the enzyme will be combined with the *carA, carAc*, and *carAd* genes of *Sphingomonas* sp. GTIN11 to form a new metabolic pathway enabling the selective cleavage of both C-N bonds in carbazole.

The most important goal of enrichment culture experiments was to obtain cultures capable of utilizing 2-aminobiphenyl, since 2-aminobiphenyl-2,3-diol is not commercially available. Hydroxybiphenyl and aminobiphenyl compounds are toxic to many microorganisms and it was challenging to obtain cultures that tolerate exposure to such compounds, let alone are capable of degrading them.

We have succeeded in obtaining a mixed culture, SL1-5, that is capable of utilizing 2-aminobiphenyl as a sole nitrogen source. This is a major accomplishment that is important to the success of the project. Obtaining a culture capable of utilizing 2-aminobiphenyl as a sole nitrogen source has proven to be quite difficult and, despite several months of enrichment culture experiments, this is the first mixed culture we have obtained that is confirmed to utilize 2-aminobiphenyl as a sole nitrogen source. The ability of SL1-5 to utilize 2-aminobiphenyl as a sole nitrogen source has been confirmed by at least six independent experiments, all of which showed the expected results for nitrogen-free and nitrogen-added controls. The ability to utilize the nitrogen present in 2-aminobiphenyl implies that the C-N bond in 2-aminobiphenyl is being cleaved. SL1-5 also utilizes 2-aminobiphenyl as a sole carbon source, but it does not utilize biphenyl as a carbon source. This suggests that SL1-5 recognizes 2-aminobiphenyl as a substrate but does not recognize biphenyl. Therefore the presence of the amino group (the C-N bond) in 2-aminobiphenyl is important to SL1-5 in the metabolism of 2-aminobiphenyl, but that the culture continues to degrade 2-aminobiphenyl so that it can also serve as a carbon source. The initial biochemical attack on 2-aminobiphenyl by SL1-5 is either to cleave the C-N bond, or to cleave/oxidize one of the C-C bonds adjacent to the C-N bond.

So far it has not been possible to obtain a pure culture derived from SL1-5 that can utilize 2-aminobiphenyl as a sole nitrogen source. This could be because a mixed culture is required for activity or simply because we have not yet isolated the appropriate pure culture. Further analysis of SL1-5 to obtain a pure culture capable of utilizing 2-aminobiphenyl will be a key focus of research in the near future. Once a pure culture is obtained then biochemical tests will be performed to determine the metabolic pathway for the metabolism of 2-aminobiphenyl, and

microbiological experiments will be performed to determine the substrate range for organonitrogen compound utilization.

In the event that a pure culture capable of utilizing 2-aminobiphenyl is not obtained the mixed culture can still be used to isolate the gene(s) that encode for a deaminase capable of selectively cleaving the C-N bond in 2-aminobiphenyl. This can be accomplished by creating a derivative of *Sphingomonas* sp. GTIN11 in which the *carB* and/or *carC* genes are deleted and then using such a strain to screen genomic libraries prepared from 2-aminobiphenyl degrading pure or mixed cultures. Currently *Sphingomonas* sp. GTIN11 can utilize carbazole as a sole source of nitrogen for growth only because nitrogen is liberated in the course of overall degradation of carbazole. If a derivative of *Sphingomonas* sp. GTIN11 were created that had functional *carA* genes but lacked *carB* and/or *carC* genes then carbazole would be converted to 2-amionobiphenyl-2,3-diol but the nitrogen would not be removed from the molecule and made available for growth of the bacteria. However, a bacteria that had functional *carA* genes but lacked *carB* and/or *carC* genes could use carbazole as a sole nitrogen source if it were provided with a deaminase capable of cleaving the C-N bond in 2-amionobiphenyl-2,3-diol. This concept will be elaborated on in the section of this report concerning genetic studies.

Genetic Studies

The reason that the isolation of a culture capable of utilizing 2-aminobiphenyl as a sole nitrogen source is a focus of enrichment culture experiments is because we ultimately want to construct a new metabolic pathway for the selective cleavage of both C-N bonds in carbazole. The first step in the carbazole degradation pathway accomplishes the cleavage of the first C-N bond in carbazole and yields a product 2-aminibiphenyl-2, 3-diol. Therefore an enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl (since 2'-aminobiphenyl-2,3-diol is not commercially available) will be useful for the cleavage of the second C-N bond in carbazole. Genetic studies of the carbazole degradation pathway encoded by *Sphingomonas* sp. GTIN11 are essential to allow us to ultimately construct a novel pathway for the selective cleavage of both C-N bonds in carbazole and to facilitate the isolation of enzymes needed to complete this pathway.

Previous research by GTI scientists resulted in the isolation of the carbazole-degrading culture *Sphingomonas* sp. GTIN11, and the cloning and sequencing a portion of the carbazole degrading genes of this culture. Prior to this project the *carA* and *carAc* genes of *Sphingomonas*

sp. GTIN11 had been cloned and sequenced, but in order to have a fully functional carbazole 1,9a-dioxygenase enzyme (CarA) the *carAd* gene encoding the ferredoxin reductase gene is also needed. In this project then we attempted to clone and sequence the *carAd* of *Sphingomonas* sp. GTIN11. DNA fragments contiguous with the *carA* and *carAc* genes were obtained by chromosome walking and eventually DNA sequence data was obtained for 5 kb in the vicinity of these *carA* and *carAc* genes. However, the *carAd* gene was not found in the vicinity of the *carA* and *carAc* genes in *Sphingomonas* sp. GTIN11. It is likely that the ferredoxin reductase of some other enzymatic pathway, encoded elsewhere on the chromosome, is capable of interacting with the products of the *carA* and *carAc* genes to produce a fully functional CarA enzyme. A map of the *Sphingomonas* sp. GTIN11 carbazole degradation operon is given below in Figure 3. While this work was in progress the DNA sequence of the *car* operon of a new carbazole-degrading culture, *Sphingomonas* sp. KA1 was published (Accession number AB095953.1) and it turns out that the DNA sequences of the *car* genes in *Sphingomonas* sp. KA1 and *Sphingomonas* sp. GTIN11 are identical.

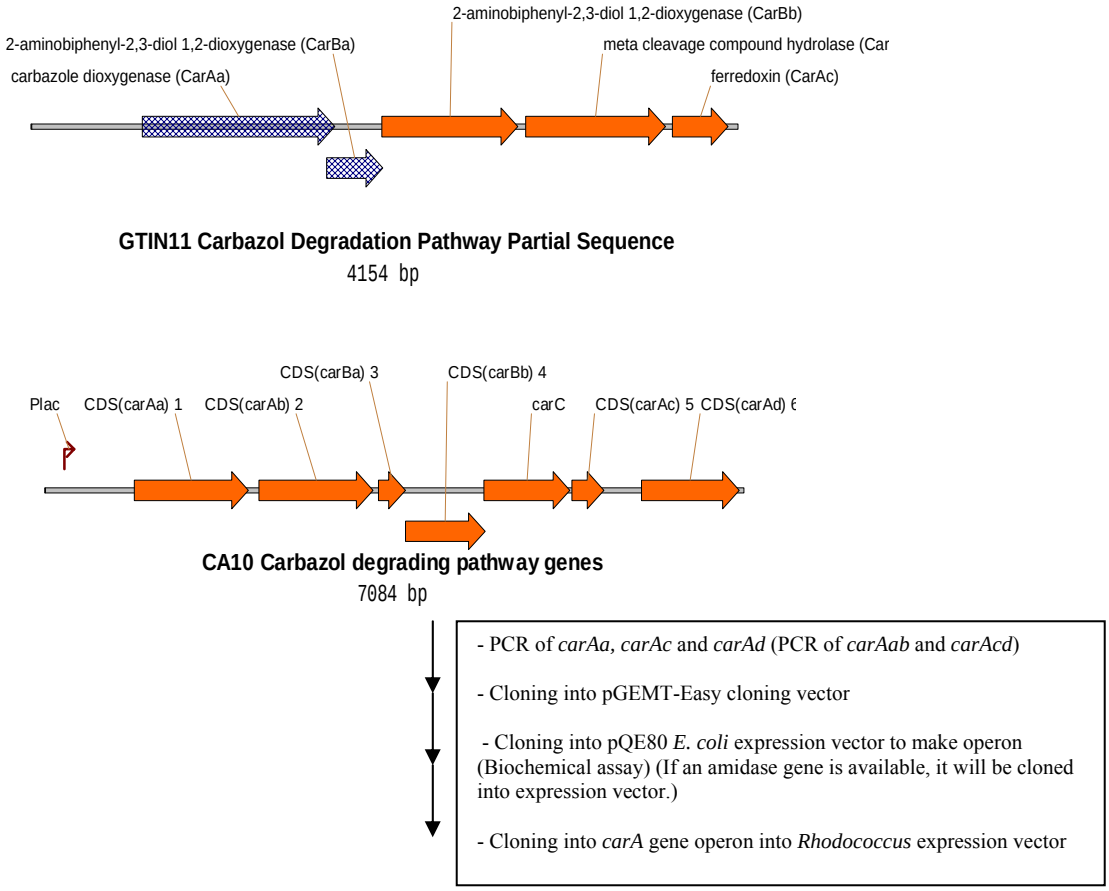
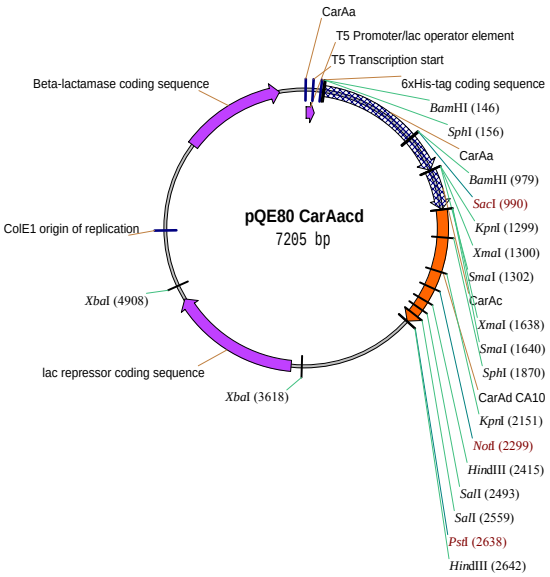


FIGURE 3. MAPS OF THE CARBAZOLE DEGRADATION OPERONS OF SPHINGOMONAS SP. GTIN11, AND PSEUDOMONAS RESINOVORANS CA10

The maps of the carbazole degradation operons of *Sphingomonas* sp. GTIN11, and *Pseudomonas resinovorans* CA10 are given in Figure 3. It is obvious that the gene arrangement of the carbazole operons in the *Sphingomonas* versus the *Pseudomonas* cultures is not identical. Not only is the gene arrangement different, but the DNA sequences vary considerably as well. The percentage similarity between the amino acid sequences of the CarAa and CarAc proteins from *Sphingomonas* sp. GTIN11, and *Pseudomonas resinovorans* CA10 are 60% and less that 40% respectively. Therefore, while the CarA enzymes from these two species catalyze the same reaction they are rather different proteins. Accordingly, in this project we have made derivative clones containing the *carA* genes from both *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10 in expression vectors both

for *E. coli* and for *R. erythropolis*. Since the *carAd* gene of *Sphingomonas* sp. GTIN11 could not be identified the *Sphingomonas* sp. GTIN11 *carAa* and *carAc* genes were combined with the *carAd* gene from *Pseudomonas resinovorans* CA10 to constitute the full complement of *carA* genes. Similarly, derivative clones containing only the *carAa, carAb, carAc*, and *carAd* genes from *Pseudomonas resinovorans* CA10 were constructed. These CarA expression vectors are devoid of functional genes for subsequent enzymatic steps in the carbazole degradation pathway so that carbazole will be converted to 2'aminobiphenyl-2,3-diol but will not be degraded further. These CarA expression constructs will be combined with suitable deaminase genes, once they are available, so that a new metabolic pathway allowing for the complete, but selective, removal of nitrogen from carbazole can be constructed. Figure 4 depicts the vectors pQE80-CarAacd and pQE80-CarAabcd-CA10, which are *E. coli* expression vectors that contain *carA* genes from *Sphingomonas* sp. GTIN11 and from *Pseudomonas resinovorans* CA10 respectively. A protein gel is also shown illustrating the expression of CarA proteins from these constructs. While protein expression experiments have demonstrated expression of the CarA proteins in some of these constructs, more work is required to verify the expression and the functionality of the CarA enzyme. Similar constructs were also made with other *E. coli* vectors such as pACYCDuet and an example of that is shown in Figure 5.



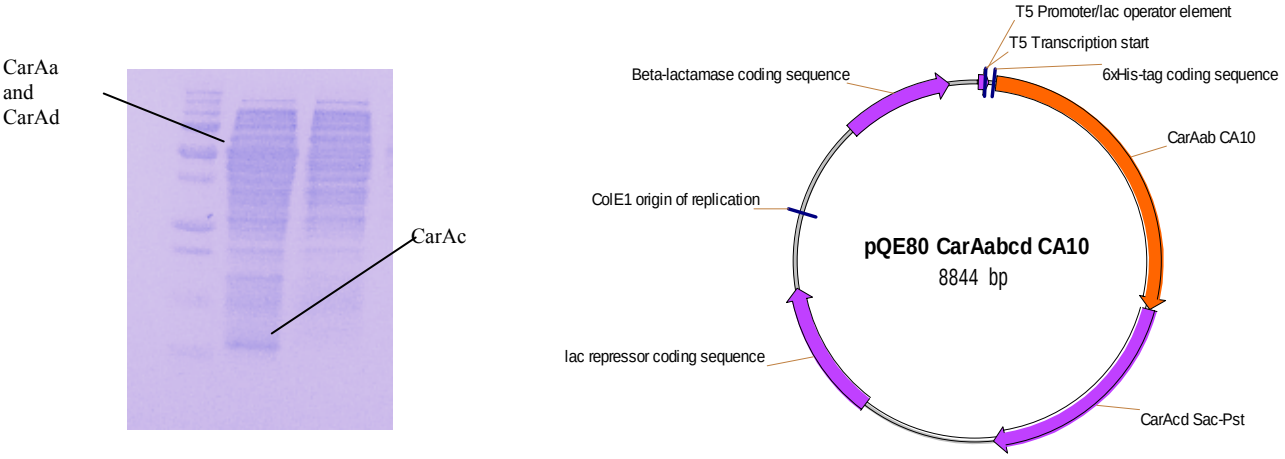


FIGURE 4. CONSTRUCTION OF *E. COLI* EXPRESSION VECTORS CONTAINING THE *CARA* GENES FROM *SPHINGOMONAS* SP. GTIN11 AND FROM *PSEUDOMONAS RESINOVORANS* CA10, AND A PROTEIN GEL ILLUSTRATING THE PRODUCTION OF THE CARA PROTEINS.

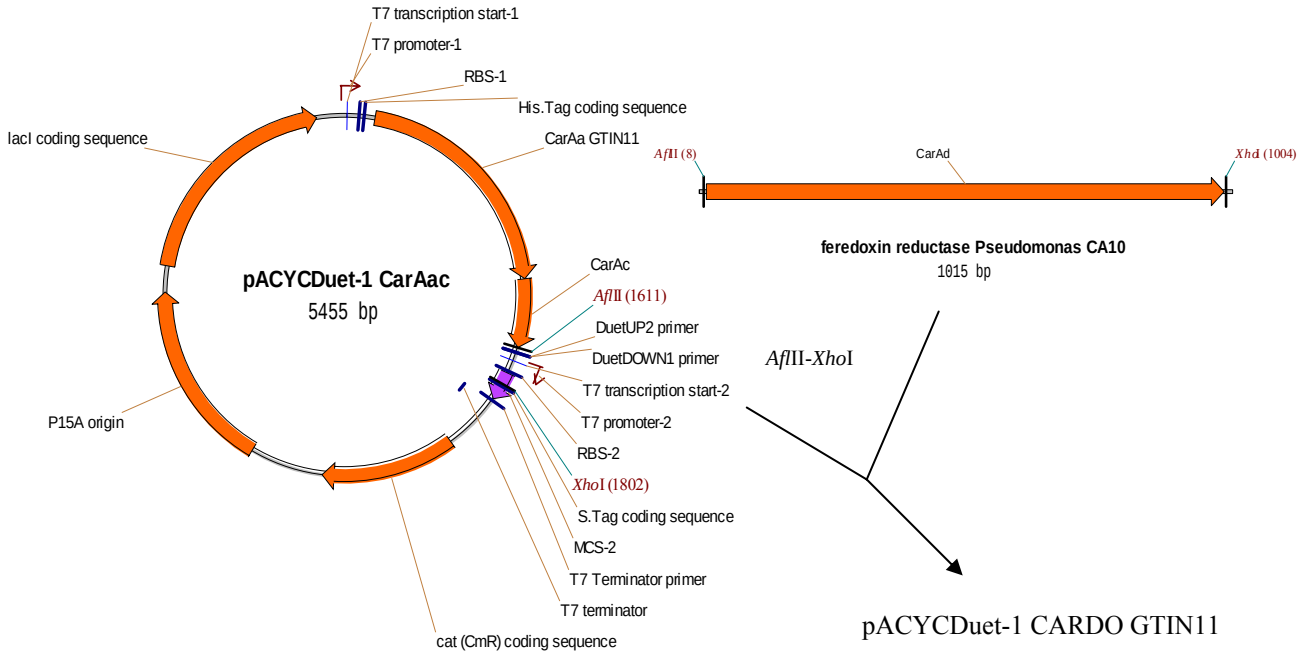


FIGURE 5. CONSTRUCTION OF PACYCDUET-1-CARDO-GTIN11 THAT CONTAINS THE *SPHINGOMONAS* SP. GTIN11 *CARA* GENES IN AN *E. COLI* EXPRESSION VECTOR.

Inactivation of *carB* in *Sphingomonas* sp. GTIN11

Sphingomonas sp. GTIN11 contains a full carbazole degradation pathway that allows this culture to utilize carbazole as a sole source of carbon as well as nitrogen. For the selective cleavage of C-N bonds we are interested only in the first step of the carbazole degradation pathway in which carbazole oxidoreductase, encoded by the *carAacd* genes, converts carbazole to 2-aminobiphenyl-2, 3-diol. The genes in the subsequent carbazole degradation pathway, such as *carB*, are not relevant for our purposes and interfere with the construction of a pathway for the selective cleavage of both C-N bonds in carbazole. One approach to avoiding the unwanted portion of the carbazole degradation pathway is to clone only the *carAacd* genes, and this has been done as described above. However, an alternative approach is to inactivate the *carB* genes in *Sphingomonas* sp. GTIN11. The approach of inactivating the *carB* genes has the advantage that it will be certain that the *carAacd* genes will be functionally expressed in this host while

there is some uncertainty if another bacterial host is used. Accordingly, we have cloned the *carBa* and *carBb* genes from *Sphingomonas* sp. GTIN11 in the *E. coli* vector pUC19. We then deleted a large portion of the *carBa* gene and replaced it with the kanamycin resistance gene (complete with its promoter) from *Rhodococcus* cloning vector pSRKgfp. The resulting plasmid, pUCcarB::Kan, is incapable of replicating in *Sphingomonas*, but it contains a kanamycin resistance gene cassette flanked by several hundred basepairs of DNA homologous to the *carBa* gene of *Sphingomonas* sp. GTIN11. Homologous recombination of the *carB* DNA sequences flanking the kanamycin resistance gene should allow pUCcarB::Kan to integrate into the chromosome of *Sphingomonas* sp. GTIN11 yielding a derivative that is kanamycin resistant, lacks a functional *carB*, but retains functional *carAacd* genes. We are in the process of utilizing pUCcarB::Kan to obtain a kanamycin resistant derivative of *Sphingomonas* sp. GTIN11 that will subsequently be characterized biochemically and genetically to confirm that that it is CarA positive and CarB negative. This derivative of *Sphingomonas* sp. GTIN11 that lacks CarB will be useful to serve as a host to clone the genes responsible for 2-aminobiphenyl degradation from SL1-5. Accordingly, chromosomal libraries of DNA obtained from the mixed culture SL1-5 (or a pure culture derived from it) can be introduced into the *carB*-defective GTIN11 strain and transformants can be selected for growth with carbazole and/or 2-aminobiphenyl as the sole source of nitrogen. In this way the genes responsible for 2-aminobiphenyl utilization in SL1-5 can be identified.

Genetic Transformation of Rhodococcus, Sphingomonas, and Pseudomonas

It is important that we place genes relevant to C-N bond cleavage into *Rhodococcus erythropolis* hosts to check the expression/functionality of those genes, and also to obtain bacterial cultures that can cleave C-S as well as C-N bonds. Similarly, the most useful cultures that we have found thus far that encode enzymes relevant to the cleavage of C-N bonds are the quinoline-metabolizing *Pseudomonas ayucida* IGTN9, and the carbazole-metabolizing *Sphingomonas* sp. GTIN11, so it is also important that we develop the ability to perform genetic manipulations of these cultures. We have several *R. erythropolis* strains: IGTS8, SQ1, CPE648 and CW25. Each of these has been used in electroporation experiments to introduce plasmids containing genes relevant to C-N bond cleavage. The following constructs have been made: IGTS8/pYgalK2-TriA, SQ1/pEBC26-Cardo, IGTS8/pEBC26-Cardo, SQ1/pYgalK2-QorMSL,

CPE648/pYgalK2-TriA, CW25/pygalK2-TriA, CPE684/pEBC26-Cardo, CW25/pEBC26-Cardo, and SQ1/pYgalK2-TriA. These and similar plasmids were also obtained in *E. coli*, and the expression of genes encoding enzymes relevant to C-N bond cleavage was investigated using polyacrylamide gel electrophoresis. The results of a typical experiment are shown in Figure 6, which illustrates the expression of the CarAa protein in *E. coli*. Future research will have to be performed to see if expression of *triA* in *E. coli* can be demonstrate and if the expression of these genes in *Rhodococcus* can be demonstrated.

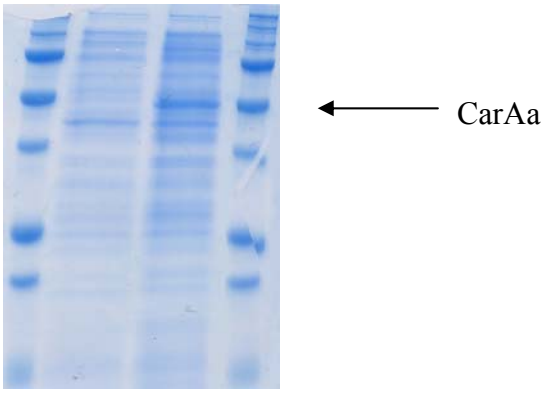


FIGURE 6. THE EXPRESSION OF THE CARAA PROTEIN IN AN *E. COLI* HOST CONTAINING PQE80-CARAA IS SHOWN.

(The arrow indicates the expected location of the CarAa protein that can be clearly seen in lane 3. Lanes 1 and 4 contain molecular weight standards.)

Genetic studies of *Pseudomonas ayucida* IGTN9 or *Sphingomonas* sp. GTIN11 have never been performed in the past so no protocols are available, nor is it known what antibiotic resistant markers or replicons can be used in these hosts. Accordingly, we performed electroporation experiments with several plasmid vectors in our possession to identify plasmids and genetic markers that may be used in these hosts. The kanamycin resistance gene encode by pKT230 appeared to be successfully transformed into both *Pseudomonas ayucida* IGTN9 and *Sphingomonas* sp. GTIN11. Once it is confirmed that we have a selectable genetic marker and a replicon that can be used in these hosts, genetic experiments can be performed.

Gibbs Assay to Investigate Substrate Range for C-N Bond Cleaving Enzymes

Investigations of biodesulfurization of the aromatic sulfur compound dibenzothiophene benefited from the use of the Gibbs assay. The Gibbs assay employs 2,6-dichloroquinone-4-chloroimide to react with aromatic hydroxyl groups and form colored products that can be

measured spectrophotometrically. It is not yet known if the Gibbs assay can be used to measure reaction products resulting from the metabolism of organonitrogen compounds, so that was investigated. Various compounds related to the metabolism of organonitrogen compounds were tested to determine if they would react with the Gibbs reagent. Model compounds tested included: 4-hydroxybenzoic acid, 4-hydroxycoumarin, 3-hydroxy isoquinoline, 3,4-dihydroxybenzoic acid, dihydroxyfumaric acid hydrate, 2-hydroxybiphenyl, 2-aminobiphenyl, 4,4'-dihydroxybiphenyl, 2,2'-dihydroxybiphenyl, coumarin, catechol, carbazole, and quinoline. All of the compounds that contain an aromatic hydroxyl group produced a reaction, but the color of the reacted products varied and blue, purple, brown, gray, and orange reaction products were observed. The carbazole and quinoline substrates did not react.

To test the use of the Gibbs assay to investigate the substrate range of C-N bond cleavage enzymes, an experiment was performed with resting cells of *Pseudomonas ayucida* IGTN9 grown using quinoline. Fresh, quinoline-grown cells were washed and concentrated, then resuspended for 2 hours in MMN media containing various substrates. After the 2-hour incubation the Gibbs assay was performed and spectrophotometric scans were performed to identify absorption wavelengths that were best suited for the detection/quantification of various reaction products. IGTN9 cells without any substrate yielded a light brown color, while in the presence of quinoline a metabolite with an absorption maximum at 330 nm (light blue) was observed. In the presence of carbazole no reaction was observed, probably indicating that carbazole does serve as a substrate for the quinoline-metabolizing enzymes. In the presence of 2-aminobiphenyl, a metabolite with an absorption maximum at 418 nm (blue/green) was observed while the 2-aminobiphenyl control yielded a blue reaction with a different absorption maximum. This indicates that 2-aminobiphenyl may be metabolized by the enzymes in IGTN9 even though this compound does not serve as a sole source of nitrogen for this culture. Melamine and diphenylamine yielded no reaction products while aniline yielded a purple color with an absorption maximum at 380 nm. A control containing aniline without cells was not tested and will have to be included in future experiments.

Directed Evolution of a Deaminase/Amidase Gene

In addition to using enrichment culture experiments to obtain an enzyme capable of cleaving the C-N bond in 2-aminobiphenyl we are also trying to modify known deaminase and

amidases using directed evolution. The objective of this task is to obtain an enzyme capable of selectively cleaving the C-N bond in 2-aminobiphenyl-2,3-diol by modifying known enzymes that act on structurally similar substrates.

When this project started no suitable enzyme for the selective cleavage of C-N bonds in 2-aminobiphenyl or related compounds was known to be available. Accordingly experiments were performed with amidase and deaminase enzymes for which genetic information was available and that acted on substrates somewhat structurally related to 2-aminobiphenyl. Initially the candidate enzymes employed were the benzamide amidase from *Rhodococcus* sp. MP50 and the melamine deaminase (*triA*) from *Pseudomonas* sp. NRRL B12227. Both of these enzymes are encoded by single genes which simplifies the mutagenesis steps required in directed evolution experiments. However, neither benzamide nor melamine is sufficiently similar to the structure of 2-aminobiphenyl to make the enzymes that metabolize these compounds ideal candidates for directed evolution experiments. Eventually, it was found that the genes for the aniline dioxygenase (AtdA) of Acinetobacter strain YAA [6] were available and that this enzyme not only accomplished the dioxygenation of aniline, but also the deamination of aniline. Aniline and 2-aminobiphenyl are closely related structures so that even though the aniline dioxygenase is a multi-component enzyme encoded by five open reading frames it is a better target for directed evolution and has recently become the focus of research in this task.

The amidase from *Rhodococcus* sp. MP50 was amplified by PCR using primers based on published DNA sequence data and cloned into the *E. coli* vector pGEMT-Easy. This DNA fragment was subsequently cloned into *E. coli* expression vector pQE80, and *Rhodococcus* expression vector pYgal-K2. Although benzamide is structurally not as close to the target substrate, 2'-aminobiphenyl-2,3-diol, as desired, it was chosen as the alternative nitrogen source in the minimal media because it is a known substrate of amidase MP50 and serves as an useful substrate to test for enzyme activity. The concentrations of benzamide used in the minimal media assay ranged from 1 to 32 mM. The cells were first induced with 0.1 mM of IPTG for 1 hr before they were inoculated into the minimal media to ensure that the innoculum possessed the amidase enzyme required for transforming benzamide. The minimal media contains 0.01 mM of IPTG. This low concentration ensures that the cells will not expend too much resource producing the enzymes and will still be able to replicate.

From the results of the assay (see Figure 7) it was found that the optimal benzamide concentration for cell growth was about 8 mM. At higher benzamide concentrations, the growth of the cells slowed down. At a benzamide concentration of 32 mM, there was no growth detected, indicating that the benzamide may be toxic to the cells at high concentrations.

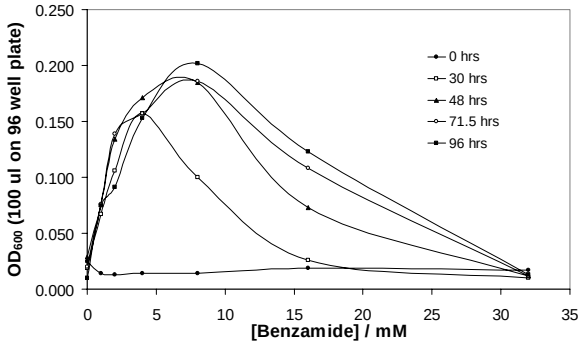


FIGURE 7. OPTICAL DENSITY OF CELLS IN MINIMAL MEDIA WITH BENZAMIDE AS SOLE NITROGEN SOURCE.

From the results of the SDS-PAGE and minimal media assay, it can be concluded that the amidase enzyme can be functionally expressed in *E. coli* DH5 alpha. By changing the nitrogen source to the desired substrate, the minimal media assay can be also used as a selection method for the directed evolution of the amidase enzyme.

The activity of amidase on 2-aminodiphenyl (2-ABP) was tested by using the minimal growth media containing 2-aminobiphenyl as the sole nitrogen source. In this set of experiments, the highest concentration of 2-ABP used was restricted to 1 mM due to the low solubility of the substrate in aqueous solution. The optical density of the cell culture is shown in Figure 8. From the figure, it can be seen that the sample containing ammonium and saturated with 2-ABP grew at a much slower rate of than that with ammonium only. This shows that 2-ABP slows the growth of the cells but is not lethal to the cells. Cells supplemented with 2-ABP as the sole nitrogen source did not show signs of growth. This might be due to two factors. Firstly, the amidase enzymes do not act on 2-ABP and hence the cells cannot catabolize 2-ABP. Secondly, the low solubility of 2-ABP (about 1.38 mM at 25^{o}C) may have resulted in the lack of nitrogen source for cell growth.

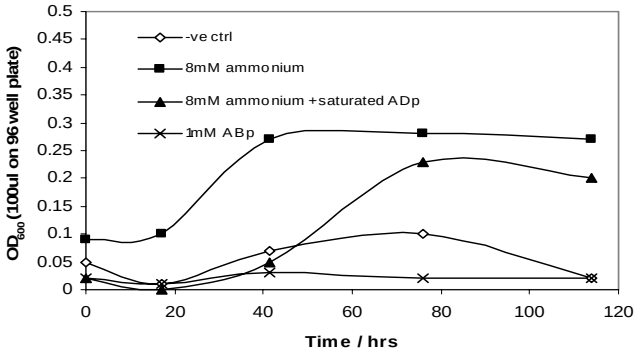


FIGURE 8. OPTICAL DENSITY OF CELLS WITH TIME IN 2-AMINOBIPHENYL MINIMAL GROWTH MEDIUM.

To engineer the amidase MP50 enzyme to hydrolyze 2'-aminobiphenyl-2,3-diol (2'-ABPD), random mutation was introduced into the amidase MP50 gene by error prone PCR (epPCR). The mutant genes were then cloned and transformed into *E. coli* DH5 alpha and put through selection on a minimal media plate with 2-aminobiphenyl (2-ABP) as the only source of nitrogen. As such, only cells producing mutant amidase enzymes capable of hydrolyzing 2-ABP to produce ammonia can obtain nitrogen necessary for growth. 2-ABP was used as a substitute for 2'-ABPD in these experiments as 2'-ABPD is not available commercially.

epPCR followed by minimal media selection was performed twice on the amidase gene but no positive clones were found. This is because 2-ABP does not possess the alpha-carbon double-bonded to an oxygen atom, as is the case of an amide. This C=O bond is essential in the amidase hydrolysis mechanism. As it is almost impossible to completely change the enzymatic mechanism of an enzyme, other candidate enzymes were investigated for use in directed evolution experiments.

The melamine deaminase encoded by the *triA* gene of *Pseudomonas* sp. NRRL B12227 is capable of selectively cleaving the C-N bonds in melamine, ammeline, and ammelide. Therefore, it may be possible to adapt this deaminase to metabolize 2-aminobiphenyl. We demonstrated that *Pseudomonas* sp. NRRL B12227 can grow in defined mineral salts media with melamine, ammeline, or ammelide serving as sole nitrogen sources. The *triA* gene was cloned into *Rhodococcus-E. coli* genetic vectors to see if the *triA* gene could confer the ability to cleave C-N bonds to an *E. coli* or *Rhodococcus* host. Most *E. coli* cultures won't grow in mineral salts media because of requirements for one or more vitamin or amino acid. However, *E. coli* strain BL21 is

a prototroph and can grow in a defined mineral salt medium, such as the MMN media used to test for the utilization of organonitrogen compounds. BL21/pYgalK2-triA was unable to utilize melamine or ammeline as sole nitrogen sources suggesting that expression of the *triA* gene in *E. coli* may be a problem. If an *E. coli* derivative can be obtained that grows with melamine as a sole nitrogen source then enrichment culture and directed evolution experiments can be performed to try to obtain a derivative of the enzyme capable of cleaving the C-N bond in 2-aminobiphenyl. The *triA* clone was also included in directed evolution experiments, but so far there are no promising enzymes obtained from directed evolution experiments. Future experiments will include examining different cloning vectors containing triA to see if functional expression can be obtained in *E. coli* and/or in *Rhodococcus erythropolis*.

The deamination of 2'-aminobiphenyl-2,3-diol (2-ABPD) by the aniline dioxygenase (AtdA), from *Acinetobacter* sp. Strain YAA was also investigated. AtdA is a multicomponent enzyme isolated from *Acinetobacter* sp. Strain YAA involved in the simultaneous deamination and oxygenation of aniline. The gene encoding for AtdA was found to have 5 open reading frames (ORFs), atdA1-A5.

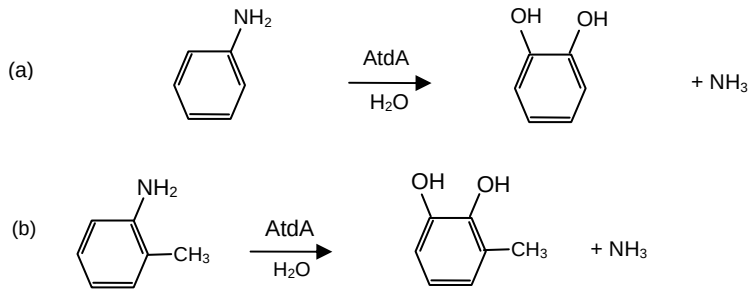


FIGURE 9. DEAMINATION AND OXYGENATION OF (A) ANILINE AND (B) O-TOLUIDINE BY ATDA.

The plasmid pAS93, consisting of the five atdA open reading frames (ORFs) cloned into a pUC19 vector, [6] 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.

Besides aniline, AtdA was found to be able to oxidize o-toluidine, which has an additional methyl side chain at the ortho position (Figure 9). As 2'-ABPD can be viewed as an

aniline molecule with a bihydroxylated phenyl attached at the ortho position, there is a potential that the AtdA enzyme can remove the amine group.

When the transformed cells were grown in a minimal media with 2-ethyl aniline (2-EA), methyl anthrinilate (MA), or 2-ABP as the sole source of nitrogen, it was found that AtdA was only able to deaminate 2-EA and use the released ammonia as a source of nitrogen (Figure 10). The larger ortho-substituent groups on ME and 2-ABP may not be able to fit into the binding pocket of the AtdA enzyme and hence no deamination activity was detected. Thus the key to engineering AtdA to accept these substrates may lie in increasing the size of the enzyme binding pocket.

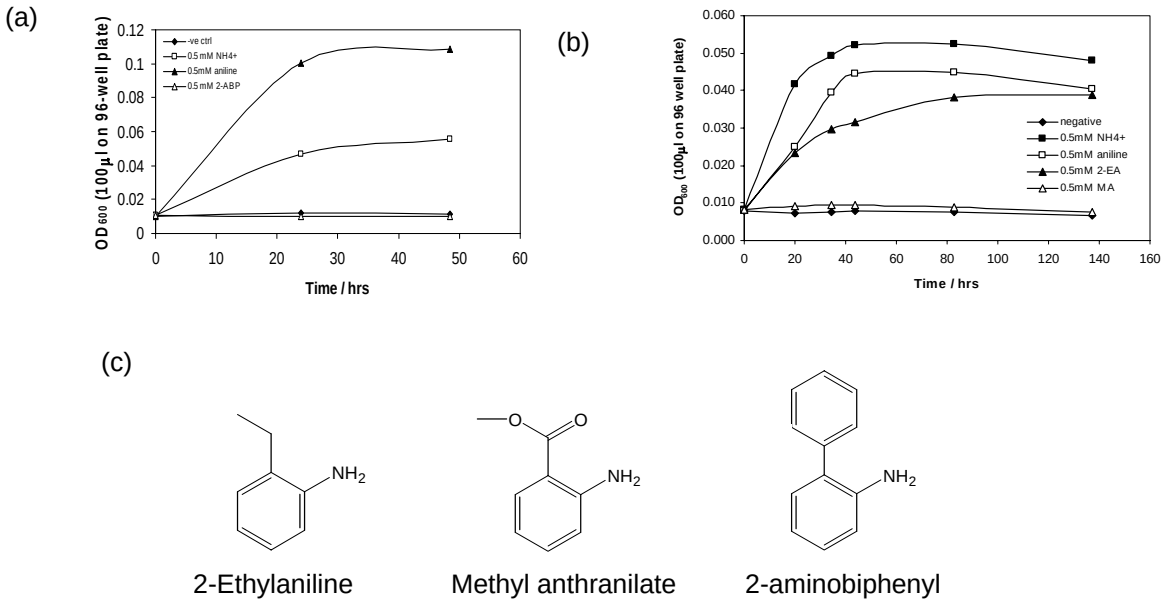


FIGURE 10. MINIMAL MEDIA ASSAY OF PAS93 TRANSFORMED *E. COLI* DH5α WITH (A) 2-ABP, (B) 2-EA AND MA. (C) CHEMICAL STRUCTURES OF 2-EA, MA AND 2-ABP.

The alpha subunits of Rieske non-heme dioxygenases often determine substrate specificity. Using the protein sequence alignment program available on the Biology Workbench (http://workbench.sdsc.edu), it was found that AtdA3 has 27.4 % homology with the alpha subunit of naphthalene 1,2-dioxygenase (NDO), a Rieske non-heme iron dioxygenase (RDO) enzyme that oxidizes naphthalene. Hence, it is highly possible that the substrate specificity of AtdA lies in the AtdA3. The crystal structure of naphthalene dioxygenase was found previously by Kauppi et al. (1998). The alignment of AtdA3 with NDO using ALIGN program on the Biology Workbench is shown in Figure 11. In the figure, the residues highlighted in blue represent the residues in NDO coordinating the Rieske center [2Fe-2S] of the enzyme. The

residues highlighted in light gray are residues in NDO coordinating the non-heme mono-nuclear iron ion in the active site. Asp205 of NDO (in dark gray) provides a direct route of electron transfer between one Rieske center of an alpha-subunit and mononuclear iron in the adjacent alpha-subunit. It was found that AtdA3 possesses all of these key residues as well. This makes NDO a suitable candidate to model AtdA3 against. Extensive modeling and simulation will be done on the AtdA3 subunit in the near future to identify key residues that can be mutated to alter the substrate specificity of aniline dioxygenase.

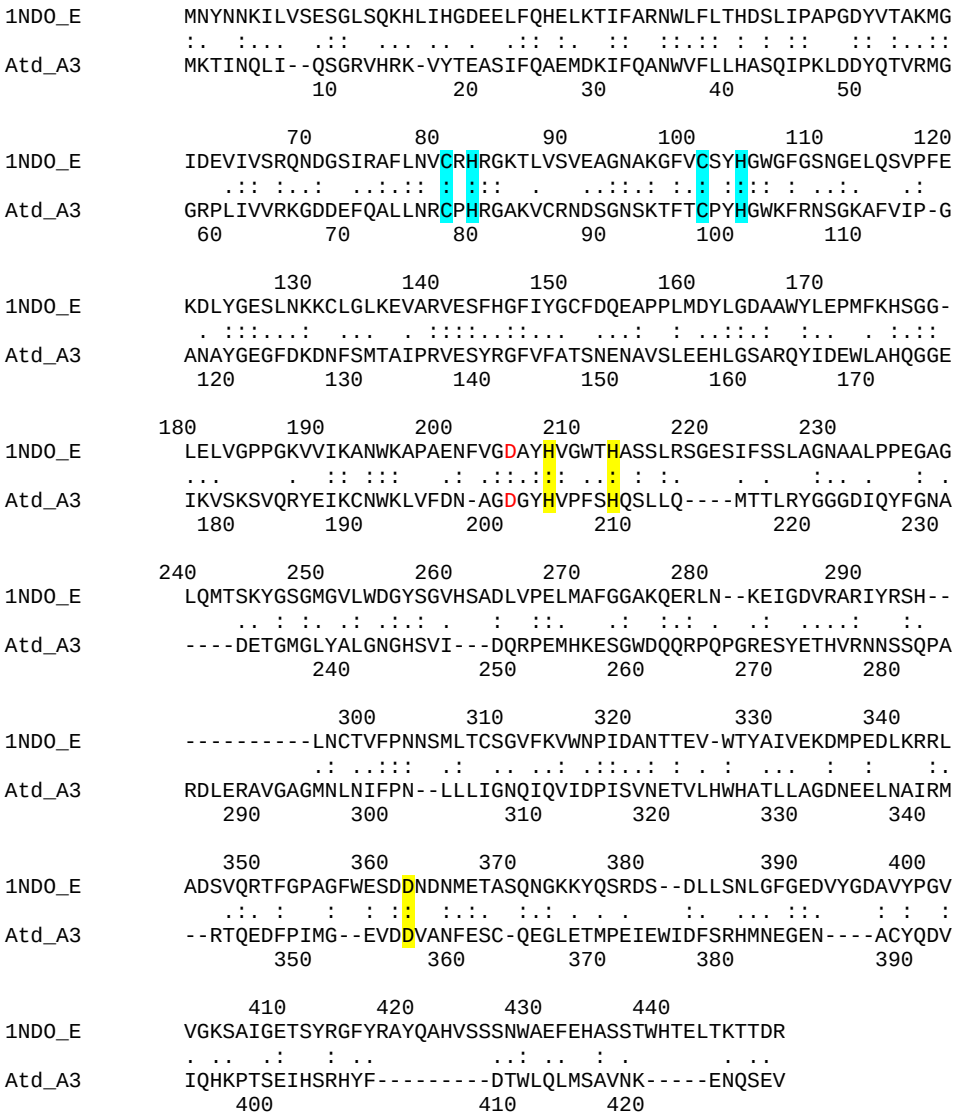


FIGURE 11. ALIGNMENT OF ATD A3 WITH NAPTHTALENE DIOXYGENASE (1NDO_E).

The next goal in this task is to relax the substrate specificity of AtdA by rational design so that the enzyme can act on larger substrate molecules such as 2-ABP and ultimately, 2'-ABPD. This can be accomplished by first using a homology modeling program, Insight II, to model the structure of AtdA against known enzyme structures. With the model, the key amino acid residues in the binding pocket of the enzyme can then be identified with other computer programs like Molecular Operating Environment (MOE). The aim is to reduce the size of the residues in the binding pocket so that larger substrate molecules will be able to enter the binding pocket. The residues in the actual enzyme can then be changed by site directed mutagenesis.

CONCLUSIONS

The *carAacd* genes that encode the carbazole dioxygenase enzyme were cloned from *Sphingomonas* sp. GTIN11 and from *Pseudomonas resinovorans* CA10. This enzyme accomplishes the cleavage of the first C-N bond in carbazole. Enrichment culture experiments have succeeded in isolating a mixed culture, SL1-5, that can utilize 2-aminobiphenyl as a sole nitrogen source. Thus this culture may be a source of an enzyme capable of selectively cleaving the second C-N bond in carbazole. However, we have not yet isolated a pure culture that has been demonstrate to contain an amidase/deaminase suitable for the cleavage of the C-N bond in 2'-aminobiphenyl-2,3-diol. Directed evolution experiments designed to obtain an enzyme capable of selectively cleaving the C-N bond in 2-ABP and 2-ABPD are now focused on the aniline dioxygenase enzyme that can deaminate the structurally similar substrate, aniline. These directed evolution experiments are proceeding well. Research in the near future will include characterization of SL1-5, continuing enrichment culture experiments to isolate novel bacterial cultures capable of utilizing aromatic organonitrogen compounds as sole nitrogen sources, and continuing experiments to express heterologous genes relevant to C-N bond cleavage in *Rhodococcus, Sphingomonas, Gordinia,* and *Pseudomonas* hosts.

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