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 will focus 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 amidase. 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.

The project is on schedule and no major difficulties have been encountered. During the first year of the project (October, 2002-September, 2003) enrichment culture experiments have resulted in the isolation of promising cultures that may be capable of cleaving C-N bonds in aromatic amides, several amidase genes have been cloned and are currently undergoing directed evolution 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.* Future research will address expression of these genes in *Rhodococcus erythropolis*. Enrichment culture experiments and directed evolution experiments continue to be a main focus of research activity and further work is required to obtain an appropriate amidase that will selectively cleave C-N bonds in aromatic substrates. Once an appropriate amidase gene is obtained it must be combined with genes encoding an enzyme capable of converting carbazole to 2'aminobiphenyl-2,3-diol: specifically *carA* genes. The *carA* genes from two sources have been cloned and are ready for construction of C-N bond cleavage pathway. 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.

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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 will focus 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 amidase. 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.

The project is on schedule and no major difficulties have been encountered. During the first year of the project (October, 2002-September, 2003) enrichment culture experiments have resulted in the isolation of promising cultures that may be capable of cleaving C-N bonds in aromatic amides, several amidase genes have been cloned and are currently undergoing directed evolution 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.* Future research will address expression of these genes in *Rhodococcus erythropolis*.

Obtaining an amidase 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. Enrichment culture experiments have succeeded in isolating microbial cultures that can utilize various organonitrogen compounds as sole sources of nitrogen needed for growth, and experiments are underway to characterize these cultures more thoroughly to confirm if any possess appropriate amidase enzymes. The most promising culture obtained thus far has been identified as *Sphingomonas yanoikuyae* by analysis of the DNA sequence of its 16S rRNA gene. The culture has been designated GTIN17 and is capable of utilizing a range of organonitrogen compounds as sole sources of nitrogen for growth: 2-aminobiphenyl, aniline, 4,4'-azodianiline, benzamide, carbazole, and para-toluidine. The ability of *Sphingomonas yanoikuyae* GTIN17 to utilize organonitrogen compounds has been determined almost exclusively thus far based on microbial growth tests in nitrogen-free media where a given organonitrogen compound serves as

the sole source of nitrogen needed for growth. Thus far we have not found a recipe for a nitrogen-free defined media that will reliably support healthy growth of *Sphingomonas yanoikuyae* GTIN17. In positive controls where an abundant supply of a carbon and an inorganic nitrogen source is supplied, the test culture should yield abundant growth, while controls that lack a nitrogen source should yield no growth. Then the ability of the culture to utilize an organonitrogen compound is determined in comparison to the amount of growth obtained in the controls. However, *Sphingomonas yanoikuyae* GTIN17 does not grow well even in the positive control so that conclusive tests of the ability of organonitrogen test compounds to support growth can't be performed. Moreover, genetic tests with PCR primers targeting *car* genes have failed to confirm the presence of carbazole degradation genes in this culture. Accordingly it is important that improved data should be obtained confirming the ability of this culture to utilize carbazole and other organonitrogen compounds as sole nitrogen sources. We have observed the best growth in defined minimal media with this culture is obtained when aniline is present, and we have determined that aniline can serve as both a carbon and a nitrogen source. The carbon sources previously used in these growth tests have been glucose, glycerol, and succinate. We are now involved in testing an expanded range of carbon compounds that include acetate, propionic acid, pyruvic acid, benzoic acid, naphthalene, biphenyl, 2,2'-dihydroxybiphenyl, and 2-phenylphenol. The use of aromatic carbon substrates is unusual, but is motivated by an attempt to identify a recipe for a defined minimal media that will reliably support healthy growth of *Sphingomonas yanoikuyae* GTIN17. In the meantime we are also continuing to perform other enrichment culture experiments to isolate cultures that possess useful C-N bond cleaving enzymes.

Another approach to the development of amidase enzymes capable of metabolizing aromatic amides is to utilize known amidase genes that encode enzymes for the metabolism of structurally related compounds and subject them to directed evolution experiments to obtain derivatives capable of cleaving C-N bonds in aromatic substrates. Directed evolution experiments are being performed with the amidase from *Rhodococcus* sp. MP50 and the melamine deaminase from *Pseudomonas* NRRL B-12227. Useful derivative enzymes have not yet been obtained, but directed evolution experiments will continue.

Once an appropriate amidase gene is obtained it must be combined with genes encoding an enzyme capable of converting carbazole to 2'aminobiphenyl-2,3-diol: specifically *carA* genes. The *carA* genes from *Sphingomonas sp*. GTIN11, and *Pseudomonas resinovorans* CA10 were

cloned on vectors capable of replicating in *Escherichia coli*. Thus the genes that encode the first enzymatic step, carbazole 1,9a-dioxygenase, have been cloned and are ready for construction of C-N bond cleavage pathway. 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.

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[1-3]. 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[1, 4-6]. 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[7].

There is currently no biochemical pathway, or thermochemical process, for the selective removal of nitrogen from compounds typically present in petroleum[1, 6]. 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[8]. 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, 3, 9]. 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[8, 10-18]. A microorganism capable of selectively cleaving C-N bonds in quinoline and removing nitrogen from petroleum was isolated and characterized[19] (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[8, 10, 11, 15-18, 20-22]. 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.

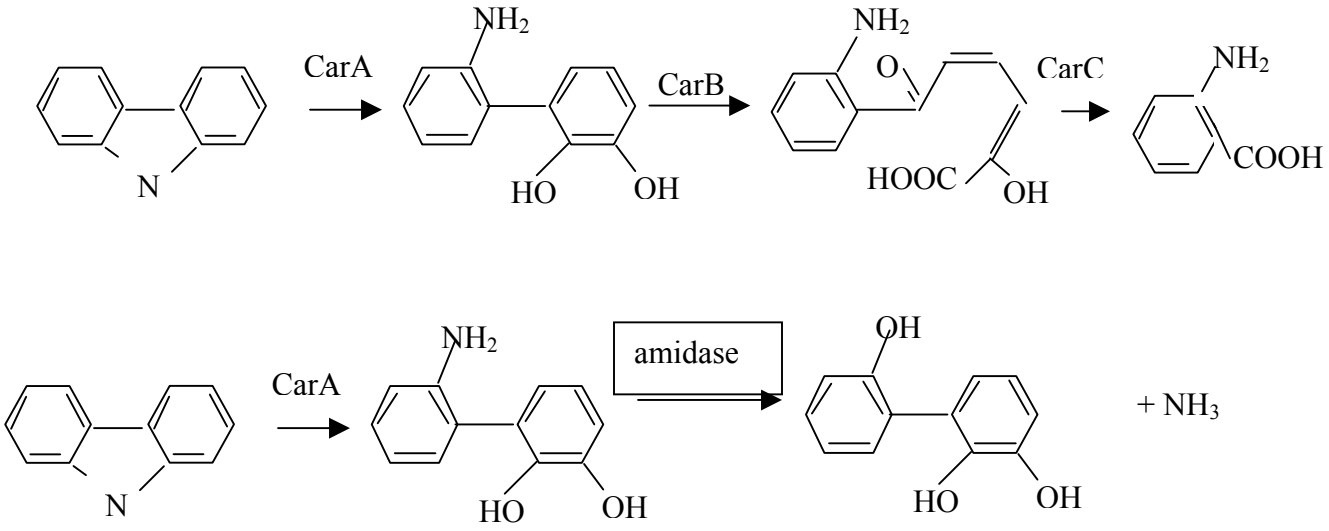


FIGURE 1. CARBAZOLE DEGRADATION PATHWAYS.

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 [8]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 amidases that can metabolize 2'-aminobiphenyl-2,3-diol and accomplish the cleavage of the final C-N bond[23, 24]. 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[11, 12, 17] 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 amidase: 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 amidases for use in directed evolution have limited ability to metabolize aromatic amides and their substrate range includes benzamide, toluamide, and anthranilamide[23, 24]. 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[4].

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[25]. Energy statistics indicate that the U.S. imports 65% of its oil demand[7]. 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[4, 7]. 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.

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						
3	Construction of pathway for C-N bond cleavage						X	X M4	X	X M5			
4	Improving the substrate range for C-N bond cleavage									X M6	X	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.

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).

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 described in the Materials and Methods section was used, but an improved media was eventually adopted that contained other essential trace elements and was thought to be more likely to result in the growth of a wider range of microbial species and increase our chances of isolating bacteria that possess useful C-N bond cleaving enzymes. 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. 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

Eventually, we succeeded in obtaining the growth of mixed cultures on various organonitrogen compounds and then streaked these mixed cultures on MMN agar plates or nutrient agar plates to obtain isolated bacterial colonies. Pure cultures were subsequently tested to determine if they could grow with organonitrogen compounds as sole sources of nitrogen and

the results obtained with the most promising cultures are summarized below along with the identification of each culture based on the analysis of the DNA sequence of its 16S rRNA gene.

Culture	MIDI Closest match
GTIN16 – *Azo degrader (azo 2-6)	*Rhodococcus globerulus*
GTIN17 – Azo degrader (from m-tol MGP 10)	*Sphingomonas yanoikuyae*
GTIN18 – Carbazole degrader (from m-tol MGP 10)	*Methylobacterium radiotolerans*
GTIN21 – Carbazole degrader (from azo 2-6)	*Gordonia bronchialis*
GTIN22 – Aniline degrader (An #3)	*Gordonia bronchialis*
GTIN23 – Carbazole degrader (from azo 2-6)	*Rhodopseudomonas palustris*
GTIN24 – Carbazole degrader (from m-tol MGP 10)	*Rhodopseudomonas palustris*
GTIN25 – Azo degrader (azo 2-6)	*Gordonia bronchialis*
GTIN26 – Azo degrader (azo 2-6)	*Rhodococcus globerulus*

* Azo = 4, 4'-azodianiline

Representative results obtained in nitrogen bioavailability growth tests were:

Nitrogen bioavailability test conditions (GGS = glucose/glycerol/succinate)

1. MMN + test compounds (C & N sources)
2. MMN + NH_4Cl + test compounds (C source)
3. MMN + GGS + test compounds (N soruce)
4. MMN + GGS + NH_4Cl + test compounds
5. MMN + GGS + NH_4Cl
6. MMN + GGS

<u>GTIN16</u>

- Growth on NH_4Cl, Benzamide, Azodianiline and Carbazole as nitrogen sources
- Azodianiline as test compound
- First growth on condition 4 and next on condition 2 and 4
- After 2 weeks condition 3 was grown slightly
- Azodianiline may be used as only carbon source.

<u>GTIN17</u>

- Full Growth (+++) on NH_4Cl, benzamide, Azodianiline, p-toluidine, aniline, and 2-aminobiphenyl
- Slight growth on m-toluidine (++), o-toluidine (+), carbazole (++)
- Negative control grow slightly (+)
- First growth on condition 5 and next on condition 3 and 4
- No growth on condition 2
- After 10 days, the growth was observed on condition 1
- Azodianiline may be first used as sole nitrogen source
- After the depletion of carbon source (GGS), test compound may be used as carbon source

GTIN18

- First growth on MMN + GGS + carbazole (Faster growth than NH_4Cl medium)
- Full growth on NH_4Cl (pink floating biomass)
- Slight growth on m-toluidine, p-toluidine, o-toluidine, aniline, and azodianiline
- First growth on condition on 3 & 4 and next on condition 5 &2
- They first attack on C-C bond (condition 2).
- Carbazole as sole nitrogen source (condition 3).

GTIN 20

- Same identification as GTIN17
- Growth inhibition effect with test compound (condition 4 grow faster than 5).
- Grwoth on condition 2 (No growth on GTIN17)

GTIN21

- Full Growth (+++) on m-toluidine, o-toluidine, and aniline
- Brown/pink color was developed on the growth of m-toluidine, and o-toluidine.
- Slight growth on carbazole medium (+), and NH_4Cl (+)
- Faster growth (first growth) on MMN + GGS + carbazole (condition 3) than condition 1 and 2
- Carbazole as sole nitrogen source (condition 3)

GTIN22

- Same identification as GTIN21
- Full grow on NH_4Cl, m-toluidine, p-toluidine, aniline and carbazole (+++)
- Slight growth on o-toluidine, and azodianiline (+)
- Brown/pink color was developed on the growth of m-toluidine, and p-toluidine
- Condition 1, 2, 4, and 5 grow at the same time.
- Condition 3 grow slightly and brown color development
- Carbazole may be used as carbon and nitrogen sources

GTIN23 and 24

- Same identification
- Condition 1, 2, 3, and 4 grow at the same time
- Carbazole may be used as carbon and nitrogen sources

GTIN26

- Similar growth pattern with GTIN16

Perhaps the most promising cultures are GTIN17, GTIN18, and GTIN21. Each of these cultures exhibits some ability to utilize carbazole as a nitrogen source so PCR tests were performed with primers designed to detect *carA* genes having sequences related to the *carA* genes of *Sphingomonas* sp. GTIN11, *Pseudomonas resinovorans* CA10, and *Sphingomonas* sp.

CB3; however, none of the new cultures yielded a PCR product of the expected size with any of these primer pairs. While amplifications with controls all yielded amplicons of the expected sizes, none of the three new cultures (GTIN17, GTIN18, or GTIN21) yielded any PCR amplification products suggesting that the carbazole degradation genes in these strains are unique. Another possible interpretation of the lack of products in PCR experiments with *car* gene primers is that these cultures may not actually metabolize carbazole. Growth in minimal medium needed for testing of organonitrogen compounds as sole nitrogen sources has been slow and rarely achieves high cell densities. This lends uncertainty to conclusions that sparse growth observed in a given test condition indicates true utilization of a given substrate. In future experiments additional growth tests will be performed in an effort to optimize the minimal medium used to grow these cultures and to obtain reproducible and unequivocal results concerning the ability of a culture to utilize a given organonitrogen compound.

Directed Evolution

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 amidases using directed evolution. 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. The cloned *Rhodococcus* sp. MP50 amidase gene was sent to Dr. Houmin Zhao at the University of Illinois at Urbana, who is a subcontractor on this project. Dr. Zhou is performing directed evolution experiments, which involve the mutagenesis of the amidase gene and selection for derivatives that altered substrate range. Specifically, derivative cultures that can metabolize 2-aminobiphenyl are of greatest interest.

The *triA* gene that encodes the melamine deaminase gene from *Pseudomonas* NRRL B-12227 has been successfully cloned into both the *E. coli* expression vector pDuet and *Rhodococcus* expression vector pYgalK2. This is another known amidase that may serve as a good starting point for directed evolution experiments leading to the development of an enzyme capable of cleaving the C-N bond in 2-aminobiphenyl. The *triA* clone was also provided to Dr. Zhou for inclusion in directed evolution experiments, but so far there are no promising enzymes obtained from directed evolution experiments.

Genetic Studies

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.

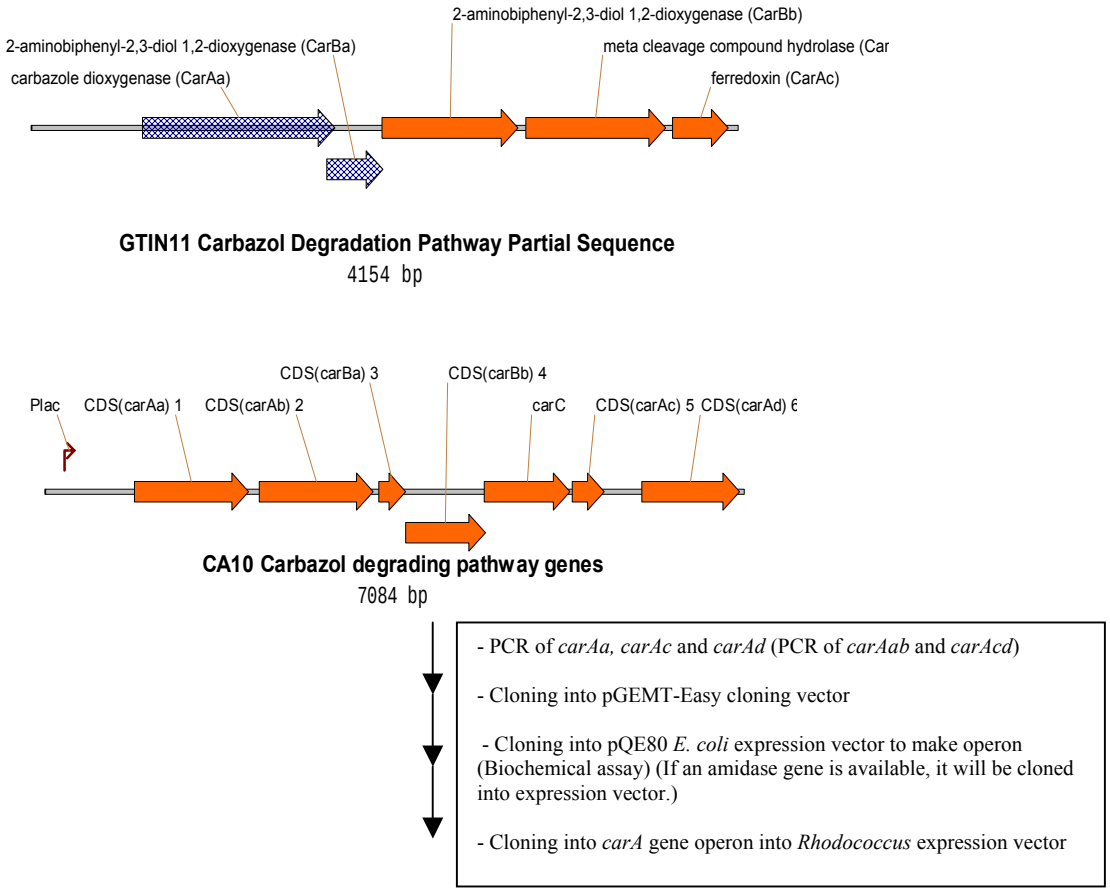
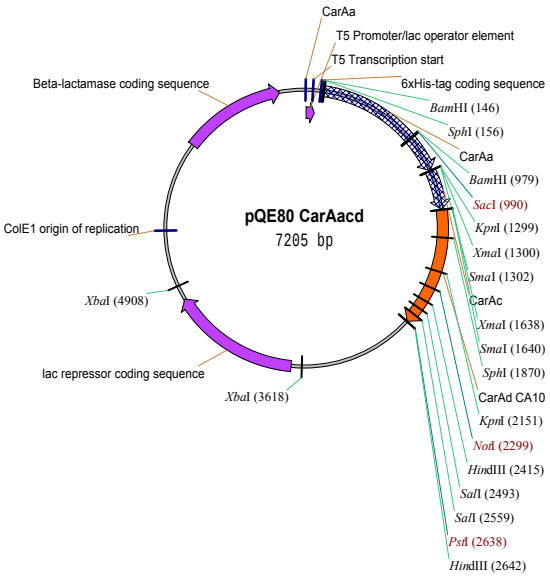


FIGURE 3. MAPS OF THE CARBAZOLE DEGRADATION OPERONS OF *SPHINGOMONAS* SP. KA1, *SPHINGOMONAS* SP. GTIN11, AND *PSEUDOMONAS RESINOVORANS* CA10

The maps of the carbazole degradation operons of *Sphingomonas* sp. KA1, *Sphingomonas* sp. GTIN11, and *Pseudomonas resinovorans* CA10 are given in the figures above. 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 *carA* 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 amidase 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. Figures 4 depicts the vectors pQE80-CarAacd and pQE80-CarAabcd-CA10, which are *E. coli* expression vectors that contains *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.



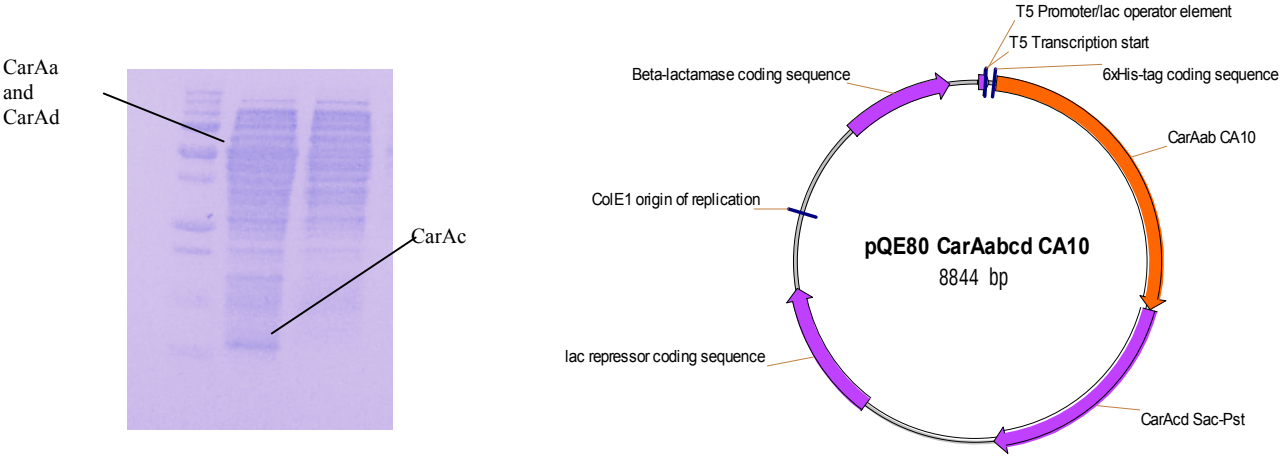


FIGURE 4. CONSTRUCTION OF *E. COLI* EXPRESSION VECTORS CONTAINING THE *CARA* GENES FROM *SPHINGOMONAS* SP. GTIN11 AND FROM *PSEUDOMONAS RESINOVORANS* CA10, AND A PROTEIN GEL ILLUSTRATING THE PRODUCTION OF THE CARA PROTEINS.

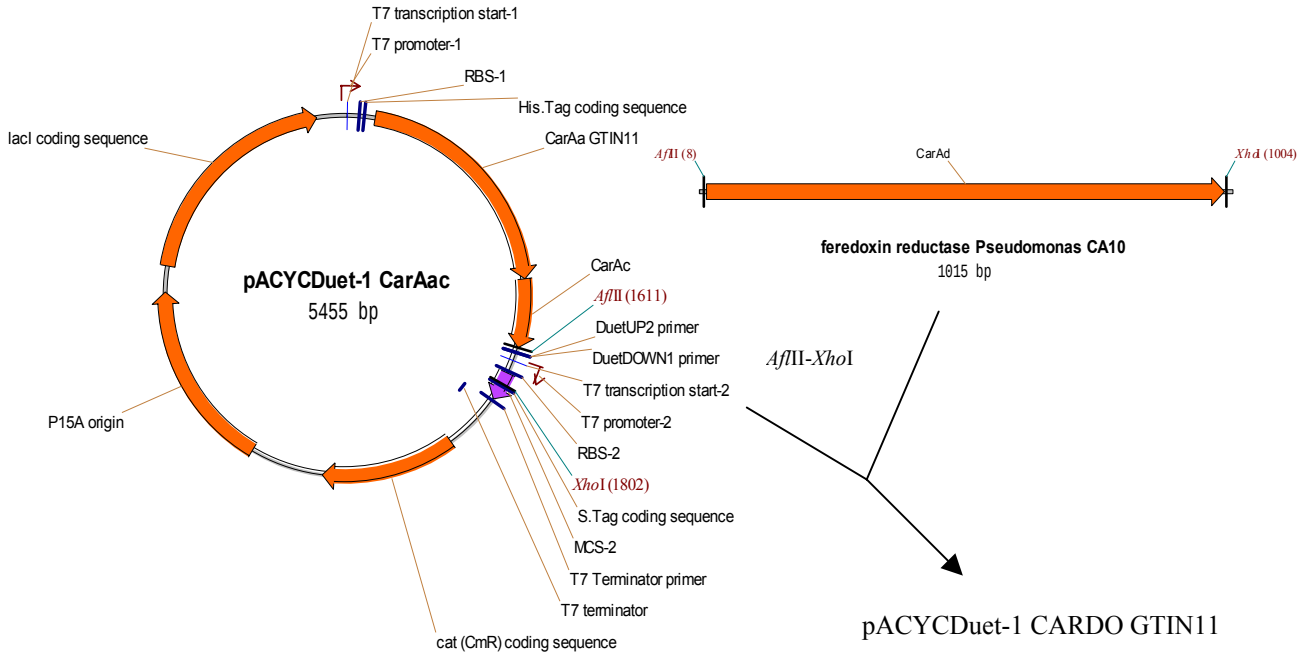


FIGURE 5. CONSTRUCTION OF PACYCDUET-1-CARDO-GTIN11 THAT CONTAINS THE *SPHINGOMONAS* SP. GTIN11 *CARA* GENES ON AND *E. COLI* EXPRESSION VECTOR.

CONCLUSIONS

The project is proceeding on schedule. This indicates that the experimental approach taken in this project appears to be appropriate to address project objectives. Enrichment culture experiments have succeeded in isolating cultures that can utilize various organonitrogen substrates as sole nitrogen sources and may contain an amidase suitable for the cleavage of the C-N bond in 2'-aminobiphenyl-2,3-diol. The *carA* genes that encode for the carbazole 1,9a-dioxygenase that catalyzes the conversion of carbazole to 2'-aminobiphenyl-2,3-diol have been cloned from *Sphingomonas* sp. GTIN11 and *Pseudomonas resinovorans* CA10 into *E. coli* expression vectors so that they can be used to construct a new C-N bond cleavage operon once an appropriate amidase gene is obtained. Future research will include continued enrichment culture experiments to characterize novel cultures until an amidase capable of cleaving the C-N bond in 2'-aminobiphenyl-2,3-diol is obtained. Additionally gene expression work with *Rhodococcus erythropolis* will be initiated, as this host may be the most appropriate for use in treating petroleum.

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