

7-2008

Sequencing, sub-cloning, expression and purification of 2-hydroxychromene-2-carboxylate isomerase from *Sphingomonas paucimobilis* EPA505

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SEQUENCING, SUB-CLONING, EXPRESSION AND PURIFICATION OF
2-HYDROXYCHROMENE-2-CARBOXYLATE ISOMERASE FROM
Sphingomonas paucimobilis EPA505

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Microbiology

by
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August 2008

Accepted by:
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ABSTRACT

Polyaromatic hydrocarbons (PAHs) are ubiquitous toxic pollutants that are slow to degrade naturally and costly to remediate artificially. Several species of bacteria have been shown to degrade PAHs, including *Sphingomonas paucimobilis* EPA505. The aerobic bacterial catabolism of the PAH naphthalene has been studied in several PAH metabolizing bacteria but never in EPA505. This study addresses this deficiency by identifying and characterizing one of the genes for naphthalene metabolism—*nahD*. *NahD* codes for the enzyme 2-hydroxychromene-2-carboxylate (HCCA) isomerase, which is the fourth of six enzymes in the upper catabolic pathway of naphthalene.

In this study, the gene *nahD* is identified and sequenced from EPA505. Then it is sub-cloned into *Escherichia coli* for expression and purification of HCCA isomerase. Sub-cloning was done by utilizing a pET-30 vector with a histidine tagged leader peptide and an enterokinase cleavage site at the insert cloning site. This allows for induction by isopropylthiogalactoside (IPTG), simple purification by Ni²⁺ affinity chromatography and restoration of the native enzyme form by peptide cleavage with enterokinase. The results suggest that *nahD* and naphthalene catabolism in EPA505 is very similar to PAH catabolism in other species.

DEDICATION

I would like to dedicate this thesis to my wonderful parents, Alan and Kathryn Brown. Thank you Dad and Mom for all your loving support throughout my collegiate pursuits. I will be forever indebted to your example of love and encouragement.

ACKNOWLEDGMENTS

Special thanks are due to my advisor, Dr. Tom Hughes, and to all of my committee members—Dr. Annel Greene, Dr. J. Michael Henson, and Dr. Harry Kurtz, Jr. I would not have been able to accomplish this project without their invaluable advice and the time they so willingly offered to me.

The Clemson University Genomics Institute (CUGI) has also earned many thanks for their work on all my sequencing projects. Chris Saski was especially helpful with his expertise.

I would also like to take this opportunity to thank all of my friends at Clemson University for all the support and fun times.

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT	ii
DEDICATION.....	iii
ACKNOWLEDGMENTS	iv
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
CHAPTER	
I. INTRODUCTION	1
II. LITERATURE REVIEW	3
Polycyclic aromatic hydrocarbons	3
Sphingomonads	5
Taxonomy and phylogeny	5
Habitat and ecology of sphingomonads.....	6
Biochemistry of PAH catabolism	8
Genetics of PAH catabolism	11
Advances in bioremediation	14
<i>Sphingomonas paucimobilis</i> EPA505 and <i>nahD</i>	16
Research objectives	18
III. MATERIALS AND METHODS	19
Sequencing and analyzing <i>nahD</i>	19
Sub-cloning <i>nahD</i> into <i>Escherichia coli</i>	23
Protein expression.....	30
Protein purification.....	32
IV. RESULTS	36
Sequence and analysis of <i>nahD</i>	36
Sub-cloning <i>nahD</i> into <i>E. coli</i>	38

Table of Contents (Continued)

	Page
Protein expression.....	41
Protein purification.....	44
V. DISCUSSION	47
REFERENCES	51

LIST OF TABLES

Table		Page
2.1	List of 27 PAHs from EPA's priority chemicals.....	4

LIST OF FIGURES

Figure		Page
2.1	Upper catabolic pathway of naphthalene	9
2.2	Lower catabolic pathway of naphthalene	12
2.3	PAHs utilized as growth substrates by <i>S. paucimobilis</i> EPA505	17
4.1	Gel electrophoresis of putative <i>nahD</i> from <i>S. paucimobilis</i> EPA505	36
4.2	Sequence of putative <i>nahD</i> from <i>S. paucimobilis</i> EPA505	37
4.3	Translation of <i>nahD</i> from <i>S. paucimobilis</i> EPA505	37
4.4	ClustalW2 alignment of HCCA isomerase from three species	38
4.5	Gel electrophoresis of PCR of <i>nahD</i> with T4 DNA polymerase extensions.....	39
4.6	Gel electrophoresis of colony PCR from <i>E. coli</i> NovaBlue	40
4.7	Gel electrophoresis of plasmid purification from NovaBlue recombinant	40
4.8	Gel electrophoresis of colony PCR for BL21(DE3)pLysS recombinant	41
4.9	Sequence of pET-30 recombinant between T7 promoter and T7 terminator	42
4.10	Cell density (OD ₆₀₀) of cultures for induction assay	42
4.11	SDS-PAGE of IPTG-induced and non-induced BL21(DE3)pLysS.....	43

List of Figures (Continued)

Figure		Page
4.12	SDS-PAGE of IPTG-induced and non-induced BL21(DE3)pLysS.....	43
4.13	Protein concentration of 1 mL fractions eluted from Ni ²⁺ resin column.....	45
4.14	SDS-PAGE of eluted fraction from Ni ²⁺ resin.....	45

CHAPTER ONE

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a seemingly ubiquitous soil contaminant with many toxic properties. These compounds pose a long-term presence because of low water solubility and slow degradation. The four bacterial genera comprising the sphingomonads have a unique capacity for degrading PAHs and thus have a promising future for bioremediation of these contaminants in the soil. Traditional methods of remediation involve costly excavation and incineration of contaminated soil and sediment. Bioremediation by resident microbes could be much less invasive on the ecosystem and would carry substantial economic savings with it. However, a comprehensive bioremediation scheme has yet to be outlined. Some attempts have been made at bioremediation, usually in conjunction with traditional physiochemical methods. While these bioremediation endeavors can cut costs, they cannot compete with the effectiveness of reducing PAH levels attained by incineration.

A better understanding of the genetic mechanisms guiding PAH metabolism in sphingomonads may allow successful bioremediation of the contaminants. Research efforts have already begun to unravel the biochemistry and genetics of PAH catabolism in aerobic bacteria—mainly *Sphingomonas* and *Pseudomonas*. In sphingomonads, the ability to degrade a wide range of high molecular weight PAHs and other recalcitrant xenobiotic compounds has led to a complex genetic

arrangement—possibly upwards of seventy-five genes in some species. Many gene products have been determined to have specific enzyme functions in the biochemical pathways that have already been deduced. Other genes have unknown function, and still others are predicted to be present but have not been located.

This study attempts to clarify the genetics of PAH catabolism in *S. paucimobilis* EPA505 by sequencing, sub-cloning, expressing and purifying the fourth enzyme in naphthalene catabolism—2-hydroxychromene-2-carboxylate isomerase. This enzyme catalyzes the conversion of the *cis-trans* isomers: 2-hydroxychromene-2-carboxylate to *trans-o*-hydroxybenzylidenepyruvate. It is assumed that the naphthalene catabolic pathway and other PAH catabolic pathways in EPA505 are similar to those of other species, but this has not yet been demonstrated. This study will continue research into the genetics of PAH catabolism in *S. paucimobilis* EPA505 to answer these questions.

CHAPTER TWO

LITERATURE REVIEW

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are a group of compounds consisting of two or more benzene rings fused into various orientations. There are hundreds of combinations possible, and they are generally found as a mixture. Some of the more common PAHs are naphthalene, anthracene, phenanthrene, and fluorene. The most common method of formation is the incomplete combustion of wood, coal or petroleum products. Because of their low water solubility, PAHs tend to endure in the soil for long periods of time, and they are released into aquifers slowly, causing lasting effects on the environment.

The toxic properties of PAHs on humans and animals have been fairly well documented. The most notable hazards are carcinogenic and mutagenic effects. PAHs are also suspected of causing reproductive, developmental and immunogenic problems^[44]. The U.S. Environmental Protection Agency's (EPA) National Partnership for Environmental Priorities has included 27 PAHs on its list of priority chemicals that need to be addressed^[54]. (See Table 2.1)

The Toxic Substances Control Act (TSCA) and the Emergency Planning and Community Right to Know Act (EPCRA) provide the authority for the EPA to monitor hazardous chemicals like PAHs. Industries and facilities are required to report use of PAHs for the Toxic Release Inventory (TRI). In 2004 the National Priority Chemicals Trends Report by the EPA identified 684 facilities across the

Table 2.1 List of 27 PAHs from EPA's priority chemicals.

acenaphthene	benzo(k)fluoranthene	dibenzo(a,l)pyrene
acenaphthylene	benzo(j,k)fluorene	7H-dibenzo(c,g)carbazole
anthracene	benzo(r,s,t)pentaphene	7,12-dimethylbenz(a)anthracene
benzo(a)anthracene	dibenz(a,h)acridine	fluorene
benzo(a)phenanthrene	dibenz(a,j)acridine	indeno(1,2,3-c,d)pyrene
benzo(a)pyrene	dibenzo(a,h)anthracene	3-methylcholanthrene
benzo(b)fluoranthene	dibenzo(a,e)fluoranthene	5-methylchrysene
benzo(g,h,i)perylene	dibenzo(a,e)pyrene	phenanthrene
benzo(j)fluoranthene	dibenzo(a,h)pyrene	pyrene

nation that reported PAHs^[54]. PAHs are also identified by the Priority List of Hazardous Substances for the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA), and they are one of the most common chemical hazards at Superfund sites on the National Priorities List.

The greatest total amount of PAHs is found in the atmosphere from the burning of wood and fossil fuels. These pollutants can remain airborne for long lengths of time by adsorbing to soot or other particulates^[4]. Eventually the PAHs settle out by precipitation or dry deposit^[4]. Although the greatest total amount is in the atmosphere, the concentration is quite low compared to local areas of heavy pollution usually found around certain industrial sites^[23]. It is in these areas of concentrated pollution that soil and water contamination yields the greatest concern for exposure^[29].

Common sites of heavy PAH pollution would include manufactured gas facilities, coking industrial sites and wood preserving plants that use creosote. Significant quantities of PAHs can also be found along roads and highways from vehicular emissions^[23]. Background levels of PAHs can even be found in remote

areas due to natural formation of PAHs from forest fires or volcanic ash^[23,29]. Interestingly, these background levels (median of 1mg/kg soil) can far exceed the 10⁻³mg/kg risk assessment goals for remediation^[23,29]. For this reason, remediation goals are often set to meet background levels of pollution rather than the much lower risk assessment levels.

Sphingomonads

The bacterial group “sphingomonads” refers collectively to four genera: *Sphingomonas*, *Sphingobium*, *Sphingopyxis* and *Novosphingobium*^[5,6]. One of the defining characteristics of sphingomonads is their ability to degrade aromatics—polycyclic aromatics in particular. The ability to utilize a wide variety of PAHs as sole carbon and energy sources led to the discovery of and interest in many sphingomonad species^[43,46,60]. Sphingomonads are of great interest to researchers looking for a bioremediation solution to PAH contaminants^[21], due to their biodegradative potential in this area.

Taxonomy and phylogeny

Sphingomonas paucimobilis is the type species of the family *Sphingomonadaceae* of the order *Sphingomonadales* of the class *Alphaproteobacteria*^[9]. *Sphingomonas paucimobilis* was originally classified as a *Pseudomonas*^[9,63], and the PAH catabolic metabolism of several *Pseudomonas* species (e.g. *P. putida*, *P. aeruginosa*, *P. stutzeri*, etc.)^[19] is testament to the similarity of the two genera. *Sphingomonas* was originally listed as a single genus but was then divided into the four genera previously mentioned based on

phylogenetic and chemotaxonomic analyses^[50]. *Sphingomonas* species are gram-negative and generally rod-shaped or slightly curved. Some cells have flagella as indicated by the etymology of “*paucimobilis*.” They form small (1mm), circular, domed, smooth colonies with entire margin and are yellow in color when cultured on agar media. Sphingomonads possess glycosphingolipids in their outer cell membrane rather than the usual lipopolysaccharides. All documented species are chemoheterotrophic and strictly aerobic.

Habitat and ecology of sphingomonads

Sphingomonads are found in a wide range of habitats: topsoil^[31,40], subsurface^[6,14], root systems^[51,55], rivers^[17,64], freshwater bodies^[3,30,61], marine water^[57], ocean sediments^[11] and more. Clinical specimens have also been described^[63] but are not of concern to PAH metabolism. Sphingomonads are found in habitats with the full range of nutrient concentrations—from ultraoligotrophic to copiotrophic environments. The range of conditions in which sphingomonads are able to flourish indicates their robust and diversified metabolic capabilities.

Quite often sphingomonads are found in areas that have been contaminated with PAHs or other recalcitrant organic compounds^[32]. Three species were discovered and characterized from deep subsurface sediments at the Savannah River Site in the Atlantic Coastal Plain^[6]. These three new species—*Sphingomonas aromaticovorans*, *S. stygia* and *S. subterranean*—have been documented to degrade many aromatics and other related compounds such as

benzoate, *p*-cresol, xylene, etc^[46]. Several other sphingomonads were also found at the site, but these were not novel species. *S. paucimobilis* EPA505 was discovered at a creosote contaminated site in Pensacola, Florida^[36,37]. It also was found to be capable of growth on a wide range of aromatic substrates as its sole carbon and energy source^[47]. Other strains of *S. paucimobilis* have been isolated from PCB contaminated soil^[1,59], γ -hexachlorocyclohexane contaminated soil^[8,39] and wastewater liquor^[12,15]. Species of *Sphingobium* have been isolated from various pentachlorophenol contaminated soils^[10,42], and *S. yanoikuyae* B1 was isolated from a polluted stream for its ability to utilize PAHs as the sole carbon and energy source^[16]. While there are many sphingomonads whose niche is PAH degradation, it is not the only ecological role these organisms fill, although it is the most relevant role to this topic.

Sphingomonads can also be dominant players in soil and aqueous environments apart from PAH contamination^[5]. In fact, *Sphingomonas* species were first identified in clinical specimens before its propensity for PAH catabolism was discovered^[63]. *Sphingomonas alaskensis* is a dominant species found in marine waters^[57] and many other sphingomonads are commonly found in uncontaminated soils (e.g. *S. dokdonensis*^[66] and *S. insulae*^[65]) or rhizospheres (e.g. *S. azotifigens*^[62] and *S. malt*^[51]). Microbial community analysis by 16S rRNA and Denaturing Gradient Gel Electrophoresis (DGGE) have shown more than once that sphingomonads are very common in marine^[34,60], freshwater^[56,60] and soil profiles^[31,32,60].

Biochemistry of PAH catabolism

The biochemistry for the complete aerobic degradation of some PAHs such as naphthalene and phenanthrene has been previously published^[19]. The metabolic pathway is divided into the upper and lower catabolic pathways. The upper catabolic pathway begins with naphthalene (or one of a few other PAHs) and converts it via several intermediates to salicylic acid. The lower catabolic pathway converts salicylic acid into several TCA cycle intermediates through branching pathways. Complete catabolism is only possible with a full complement of the necessary genetically coded enzymes, which not all bacteria possess.

The upper catabolic pathway for naphthalene metabolism is as follows: naphthalene is converted to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene by naphthalene dioxygenase; *cis*-1,2-dihydroxy-1,2-dihydronaphthalene is converted to 1,2-dihydroxynaphthalene by naphthalene dihydrodiol dehydrogenase; 1,2-dihydroxynaphthalene is converted to 2-hydroxychromene-2-carboxylate by 1,2-dihydroxynaphthalene dioxygenase; 2-hydroxychromene-2-carboxylate is converted to *trans*-*o*-hydroxybenzylidenepyruvate by 2-hydroxychromene-2-carboxylate isomerase; *trans*-*o*-hydroxybenzylidenepyruvate is converted to salicylaldehyde by *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase; and lastly, salicylaldehyde is converted to salicylic acid by salicylaldehyde dehydrogenase. (See Figure 2.1)

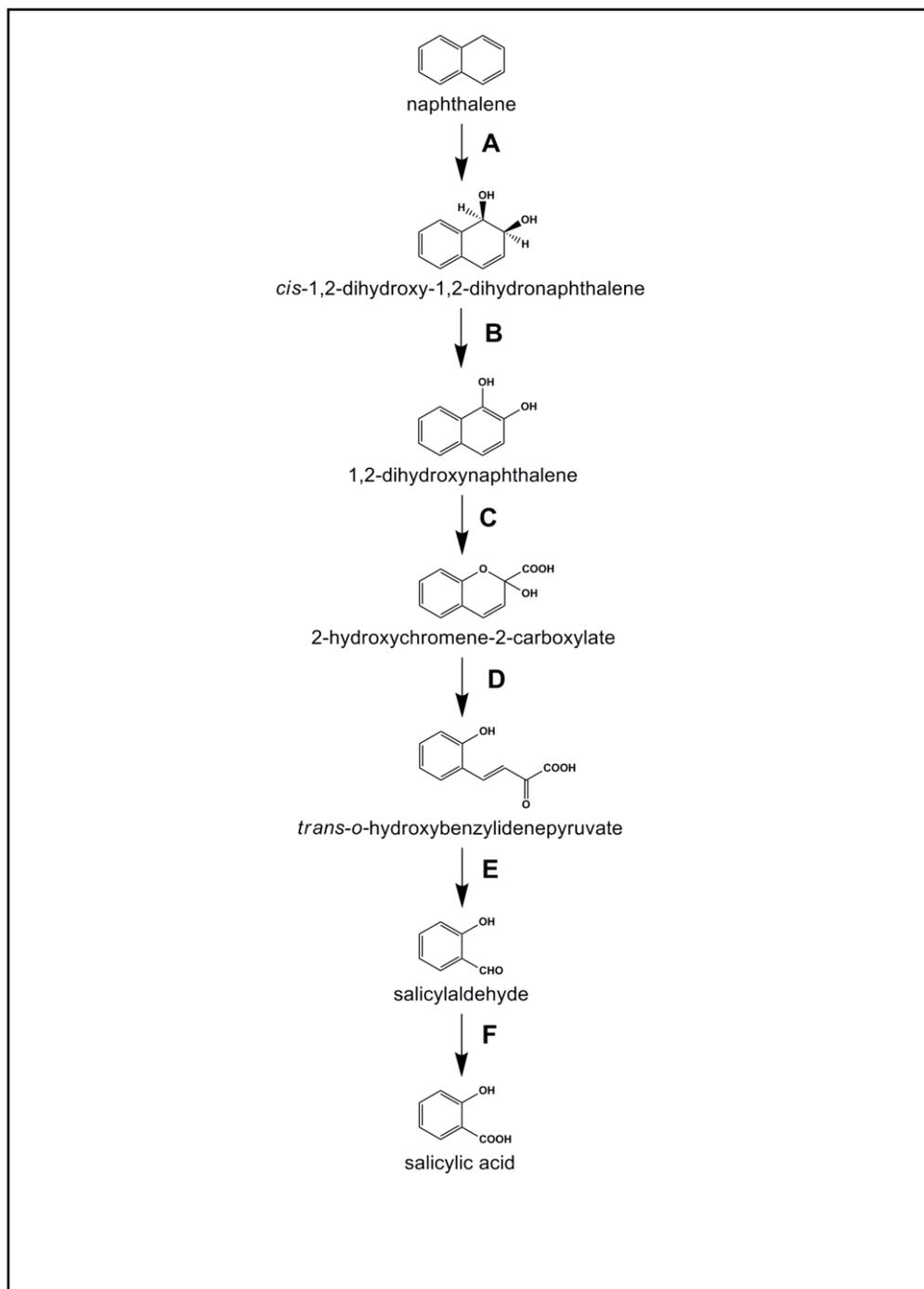


Figure 2.1: Upper catabolic pathway of naphthalene. Enzymes: (A) naphthalene dioxygenase, (B) *cis*-naphthalene dihydrodiol dehydrogenase, (C) 1,2-dihydroxynaphthalene dioxygenase, (D) 2-hydroxychromene-2-carboxylate isomerase, (E) *trans*-*o*-hydroxybenzylidenepyruvate hydratase aldolase, (F) salicylaldehyde dehydrogenase.

The first enzyme for naphthalene degradation—naphthalene dioxygenase—is actually an enzyme complex composed of four subunits: a ferredoxin-reductase, a ferredoxin and two iron-sulfur proteins. The two iron-sulfur proteins are specific for naphthalene substrate, but alternate iron-sulfur proteins may complex with the same ferredoxin/ferredoxin-reductase subunits to bind and metabolize other PAHs such as phenanthrene and anthracene^[19]. The rest of the enzymes in the upper catabolic pathway appear to work with multiple PAHs.

The branching lower catabolic pathway for aerobic PAH degradation is as follows: (1) salicylic acid is converted to gentisic acid by salicylate 5-hydroxylase; gentisic acid is converted to maleylpyruvate by gentisate 1,2-dioxygenase; maleylpyruvate is converted to fumarylpyruvate by maleylpyruvate isomerase; and lastly, fumarylpyruvate is converted to pyruvate and fumarate by fumarylpyruvate hydrolase; or (2) salicylic acid is converted to catechol by salicylate hydroxylase; catechol is converted to 2-hydroxymuconic-semialdehyde by catechol 2,3-dioxygenase; 2-hydroxymuconic-semialdehyde is converted to 2-oxo-4-pentonic acid by semialdehyde hydrolase or through two other intermediates by hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase; 2-oxo-4-pentonic acid is then converted to 4-hydroxy-2-oxovaleric acid by 2-oxopent-4-enoate; 4-hydroxy-2-oxovaleric acid is converted to pyruvic acid and acetaldehyde by 2-oxo-4-hydroxypentanoate aldolase; and lastly, acetaldehyde is converted to acetyl-CoA by acetaldehyde dehydrogenase; or (3) salicylic acid is converted to

catechol by salicylate hydroxylase; then catechol is alternatively converted to *cis,cis*-muconic acid by catechol 1,2-dioxygenase; *cis,cis*-muconic acid is converted to muconolactone by *cis,cis*-muconate lactonizing enzyme; muconolactone is converted to β -keto adipate-enol-lactone by muconolactone isomerase; β -keto adipate-enol-lactone is converted to β -keto adipic acid by β -keto adipate-enol-lactone hydrolase; β -keto adipic acid is converted to β -keto adipyl-CoA by β -keto adipate:succinyl-CoA transferase; and lastly, β -keto adipyl-CoA is converted to succinyl CoA and acetyl-CoA by β -keto adipyl-CoA thiolase. (See Figure 2.2)

The upper and lower catabolic pathways have been more thoroughly studied in *Pseudomonas*, but initial research indicates that it is the same or very similar for sphingomonads^[43]. There are also at least a few other species that catabolize PAHs, including *Comamonas*, *Burkholderia*, *Mycobacterium* and *Rhodococcus*.

The catabolic pathways for a few other PAHs such as pyrene and fluorene have also been at least partially deduced^[19,24], but will not be discussed in further detail here.

Genetics of PAH catabolism

The “classical” PAH catabolic genes for naphthalene degradation (*nah* genes) have been well studied in *Pseudomonas*. Their organization consists of three well defined operons: one for the upper catabolic genes, another for the lower catabolic genes and a third for a regulator gene^[19]. Operons for metabolism of a few other PAHs may also be present, dependent on the particular species.

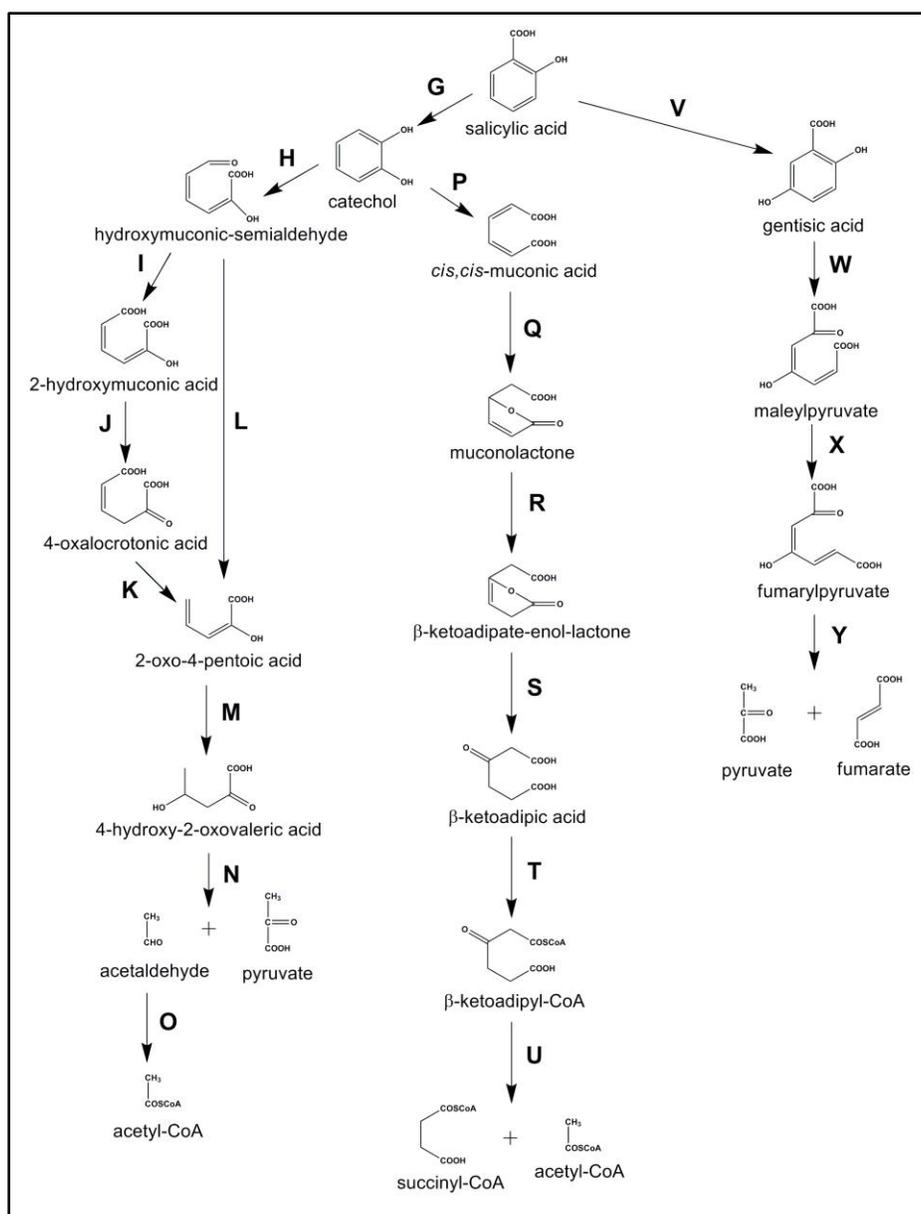


Figure 2.2: Lower catabolic pathway of naphthalene. Enzymes: (G) salicylate hydroxylase, (H) catechol 2,3-dioxygenase, (I) hydroxymuconic semialdehyde dehydrogenase, (J) 4-oxalocrotonate isomerase, (K) 4-oxalocrotonate decarboxylase, (L) hydroxymuconic semialdehyde hydrolase, (M) 2-oxopent-4-enoate hydratase, (N) 2-oxo-4-hydroxypentanoate aldolase, (O) acetaldehyde dehydrogenase, (P) catechol 1,2-dioxygenase, (Q) *cis,cis*-muconate lactonizing enzyme, (R) muconolactone isomerase, (S) β -keto adipate-enol-lactone hydrolase, (T) β -keto adipate:succinyl-CoA transferase, (U) β -keto adipyl-CoA thiolase, (V) salicylate 5-hydroxylase, (W) gentisate 1,2-dioxygenase, (X) maleylpyruvate isomerase, (Y) fumarylpyruvate hydrolase.

However, genetic organization in the sphingomonads is much more complex. Naphthalene catabolic genes are strewn into a convoluted arrangement with genes for metabolism of many other aromatics such as toluene, biphenyl, xylene, etc^[43]. Although arranged differently, the individual enzymatic genes in sphingomonads have high sequence homology to those in *Pseudomonas*.

The PAH catabolic genes of sphingomonads may be located either chromosomally located or extra-chromosomally. A 40-kb region on the chromosome of *Sphingobium yanoikuyae* was sequenced and found to contain 35 genes for catabolism of aromatic compounds^[25]. Several *Sphingomonas paucimobilis* EPA505 genes have been sequenced or partially sequenced from the chromosome^[48,49]. had The complete sequence of the 184-kb plasmid (pNL1) from *Novosphingobium aromaticovorans* F199 containing 79 genes for catabolism of aromatics was published^[45]. Significant amounts of sequencing of PAH catabolic genes have also been accomplished in *Sphingomonas chungbukensis* DJ77^[43], *Sphingobium agrestis* HV3^[67], *S. xenophaga* BN6^[28,22], and *S. sp.* P2^[43]. Much interpretation is still needed from all this sequencing, and many predicted genes are yet to be found in some species.

All of the species listed above have a high degree of homology in both nucleotide sequence and genetic organization. This could possibly be accounted for by horizontal gene transfer, because PAH catabolic genes are sometimes located on self-transmissible plasmids or adjacent to transposable elements^[19,43].

Advances in bioremediation

In order to understand the potential advantages of biological remediation, it is first necessary to briefly discuss the conventional methods usually employed for remediation and the EPA's remediation protocols for Superfund sites. Since 1980, regulation of hazardous waste sites has been under the jurisdiction of the EPA as outlined in the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) since 1980. A Hazard Ranking System evaluates and ranks hazardous waste sites for addition to the National Priorities List (NPL). Sites on the NPL then undergo a remedial investigation (RI) and a feasibility study (FS). The RI appraises the general condition and hazards/toxins present at the site, as well as a risk assessment of any toxins found. The FS recommends options for remediation. A record of decision (ROD) is then signed as the final authority for the guidelines of remediation. The ROD would include detailed instructions as to what hazards need to be remediated, where the hazards are located, what processes will be used to remediate, and what acceptable levels of remaining contamination are allowed.

RODs often implement an excavation and incineration technique if PAH contamination requires remediation beyond institutional controls or containment of the toxins. A typical excavation could remove tens or hundreds of thousands of tons of soil or other solid waste up to a few meters deep. Incineration of the soils/solid-waste would then involve the removal of moisture content from the waste in a hot kiln. Then the temperature would be increased to approximately

600-800°C to volatilize all PAHs and other toxins present. The resulting ash would be tested to ensure sufficient decontamination, and air pollution prevention and monitoring measures must also be in place. Excavation and incineration of soils and solid waste is a very effective treatment to reduce PAH levels, but it is also very expensive, costing hundreds of dollars per ton of soil or solid waste.

An alternative to incineration, which is sometimes employed, is soil washing with solvents or surfactants. Excavation of soil is still required, but operating costs to flush the waste out with washing techniques could be lower than high temperature incineration techniques. Once the PAHs have been sufficiently removed from the soil/waste, they may be disposed of in any number of ways. The downside to washing methods is that they do not remove contaminants as efficiently as incineration. Also, the solvents or surfactants often have toxic properties themselves, complicating the remediation process^[58].

Bioremediation schemes have yet to be demonstrated worthy of supplanting physiochemical methods of remediation, but continued efforts are being poured into research to achieve viability. Biodegradation of PAHs by sphingomonads or other organisms is limited to only the bioavailable portion of PAHs in the soil. Bioavailability is very low because of the hydrophobic nature of high molecular weight PAHs which cause them to adsorb to dirt particles rather than to solubilize in water^[23]. Generally only the aqueous phase is accessible to microbes. Despite this hindrance, biodegradation does occur at low rates. A more complete understanding of the microbial metabolism and physiology of PAHs may reveal

methods to enhance biodegradation.

For now, bioremediation is being implemented in conjunction with traditional methods like soil washing. For example, the contaminated soil could be excavated and washed with surfactants; then the wash, now containing PAHs, could be treated in a bioreactor^[18,68,69]. However, high costs would still be associated with such a process. Farming techniques, which add nutrients to the soil and tilling to aerate, have been used to stimulate microbial biodegradation, but only minimal reductions of toxins have been attained through these processes^[20]. The ultimate goal of bioremediation would be an *in situ* technique that would somehow stimulate biodegradation by a native microbial community. If such a scheme could reduce PAH concentrations to an acceptable level, it would most likely have tremendous cost advantages as well as being much less invasive to the ecosystem compared to an excavation processes.

Sphingomonas paucimobilis EPA505 and *nahD*

S. paucimobilis EPA505 was isolated from a creosote contaminated site, and subsequent studies have shown it capable of metabolizing many aromatic substrates. EPA505 was able to utilize the following as sole carbon and energy sources: phenanthrene, naphthalene, fluoranthene, toluene, benzoic acid, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 1-chloro-2,4-dinitrobenzene, anthracene, 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, salicylic acid and catechol^[47]. (See Figure 2.3 for compounds and structures.) The genetics of PAH catabolism for EPA505 began to be unraveled with Tn5

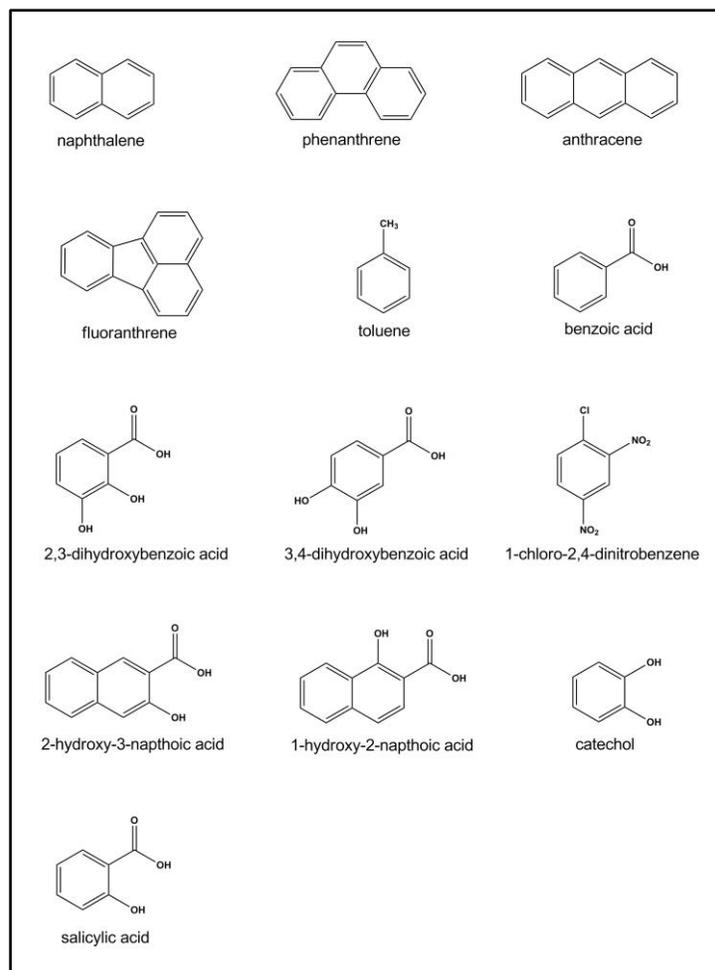


Figure 2.3: PAHs utilized as growth substrates by *S. paucimobilis* EPA505.

mutagenesis studies. These studies revealed the partial sequence of four putative genes and two promoters^[48,49]. Probes designed to hybridize with conserved region of dioxygenases followed by chromosome walking revealed the sequence of several more putative genes for PAH metabolism (unpublished data). Among those genes was a sequence resembling the fourth enzyme in the upper catabolic pathway for naphthalene degradation—*nahD* or 2-hydroxychromene-2-carboxylate isomerase (HCCA isomerase).

HCCA isomerase has been studied in at least three strains of *Pseudomonas* and one *Sphingomonas* (*P. putida*^[53], *P. putida* G7^[13], *P. sp.* TA-2^[41] and *S. yanoikuyae* B1^[26]) and the gene sequence has been published for several other sphingomonads. The enzyme catalyzes the isomerization of 2-hydroxychromene-2-carboxylate (HCCA) to *trans*-*o*-hydroxybenzylidenepyruvate (*t*HBP). It appears to be a dimeric enzyme with reduced glutathione (GSH) acting as a cofactor. Studies in *S. yanoikuyae* B1 also indicate that HCCA isomerase is also capable of catalyzing the isomerization of 1,2-dihydroxyanthracene to 2-hydroxybenzochromene-2-carboxylate in the anthracene catabolic pathway.

Research objectives

The purpose of this study was to sequence the putative HCCA isomerase gene from *S. paucimobilis* EPA505 and sub-clone it into a protein expression vector for induction and purification. Sequencing was accomplished by polymerase chain reaction (PCR) amplification with primers targeting regions just upstream and downstream of the putative *nahD* gene. Sub-cloning into *E. coli* NovaBlue™ and *E. coli* BL21(DE3)pLysS was achieved with a pET-30 Ek/LIC vector. Expression of *nahD* was by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purification was done by utilizing Ni²⁺ affinity chromatography, taking advantage of the histidine tag on the expressed HCCA isomerase.

CHAPTER THREE

MATERIALS AND METHODS

Sequencing and analyzing *nahD*

Preliminary sequence data indicated the presence of *nahD* in *Sphingomonas paucimobilis* EPA505 (unpublished data). Genomic DNA was extracted from EPA 505 for polymerase chain reaction (PCR) amplification and sequencing of the product. The nucleotide sequence and its translation were then compared to previously published sequences to determine the identity.

Stock culture of *S. paucimobilis* EPA505 was removed from -80°C storage in a glycerol-based medium (15% v/v glycerol and 85% LB culture—10 g/L Bacto™ Tryptone from Becton, Dickinson and Company (BD, Sparks, MD), 5 g/L yeast extract from Difco (Detroit, MI) and 10 g/L NaCl) and was streaked onto LB agar (15 g/L agar from Difco) and incubated at 37°C for 24 hrs. An isolated colony was inoculated into 5 mL LB and incubated overnight at 37°C with gentle agitation (60 rpm) in G24 Environmental Incubator Shaker from New Brunswick Scientific (Edison, NJ). Genomic DNA extraction from the EPA505 culture was then performed as follows with Ultra Clean™ Microbial DNA isolation kit (Catalog #12224-50) from Mo Bio Laboratories (Carlsbad, CA) according to manufacturer's instructions.

A 2 mL collection tube with 1.8 mL of culture was centrifuged at 10,000 x g for 30 seconds. The supernatant was decanted and centrifuged at 10,000 x g for another 30 seconds. The remaining supernatant was pipetted out and discarded.

The cell pellet was resuspended in 300 μ l MicroBead solution and vortexed gently to mix. Then the resuspended cells were transferred to a MicroBead tube and 50 μ l Solution MD1 was added. The bead-beating tube was then secured horizontally onto a vortex adapter and vortexed on maximum for 10 minutes to lyse the cells using the Vortex-Genie from Fisher Scientific (Bohemia, NY). The tube was then centrifuged at 10,000 x g for 30 seconds, and the supernatant was transferred to a clean 2 mL collection tube. Then 100 μ L Solution MD2 was added, vortexed briefly to mix, then incubated at 4°C for 5 minutes. The tube was then centrifuged at 10,000 x g for one minute to pellet any remaining cellular debris. The supernatant was then transferred to a clean collection tube and 450 μ L Solution MD3 was added. Tube was vortexed briefly to mix. The supernatant was loaded into the Spin Filter in two aliquots, followed by centrifugation at 10,000 x g, and the flow through was discarded. Filter-bound DNA was then washed with 300 μ L Solution MD4 and centrifuged at 10,000 x g for 30 seconds. The flow through was discarded and the tube centrifuged again for 1 minute to dry. The Spin Filter was then transferred to a clean collection tube and 50 μ L Solution MD5 was added to the center of the filter membrane followed by centrifugation at 10,000 x g for 30 seconds. The flow through was then checked for DNA concentration and purity by OD₂₆₀ and OD_{260/280} as well as agarose gel electrophoresis. The OD₂₆₀ and OD_{260/280} measurements throughout this study were performed with a Biophotometer (Cat. #95200000-4) from Eppendorf (Hamburg, Germany). Agarose gels were always 1% agarose from

Sigma Chemical (St. Louis, MO) in 1X TBE buffer (90 mM Tris-borate, 4 mM EDTA, pH 8.0) with 0.5 µg/mL ethidium bromide. Samples of 5 µL were then added to 1 µL 6X loading dye and transferred to the agarose gel. Gel electrophoresis was conducted at 150V for approximately one hour.

PCR primers were designed to target sites upstream and downstream of the putative 588 base pairs (bp) of coding sequence for amplification. Primers were designed with Primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>). The forward primer (5' TCC AGC AGA TTC AAA ACA CG 3') with T_m of 53.4°C, designated "Fred," targeted the non-coding strand approximately 350 bp upstream of the suspected start codon. The reverse primer (5' GCT TAG CGG TTT CGA TTG AC 3') with T_m of 54.2°C, designated "Renée," targeted the coding strand approximately 50 bp downstream of the suspected stop codon. The forward primer Fred and reverse primer Renée were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and diluted in nuclease-free H₂O to 10 µMol. The entire PCR amplified product should be slightly less than 1000 bp based on the preliminary sequencing data.

The PCR reactions were assembled with the following: 2 µL isolated EPA 505 DNA template, 2 µL forward primer Fred (10 µMol), 2 µL reverse primer Renée (10 µMol), 19 µL H₂O and 25 µL GoTaq® Green MasterMix (Cat. #M7122) from Promega (Madison, WI). PCR reactions were carried out in a Genius thermocycler with heated lid from Techne (Princeton, NJ). Reactions were as follows: 95°C initial denaturation for 2 minutes; 30 cycles of 95°C for 45 seconds,

58°C for 45 seconds and 72°C for 45 seconds; final elongation at 72°C for 2 minutes, and then held at 4°C.

The PCR product was then purified using QIAquick® PCR purification kit (Cat. #28706) from QIAGEN (Valencia, CA) following manufacturer's instructions as follows: The 50 µL PCR reaction was added to 250 µL Buffer PB and the mix was transferred to a QIAquick spin column in a 2 mL collection tube. The DNA was bound to the filter by centrifugation at 10,000 x g for 1 minute, and the flow through was discarded. Then the DNA was washed with 750 µL Buffer PE and centrifuged for 1 minute. Flow through was discarded, and the filter was centrifuged again for one minute to dry. The QIAquick column was then transferred to a clean 1.5 mL microfuge tube, and 50 µL of TlowE buffer (10 mMol Tris-HCl, 0.1 mM EDTA, pH8.0) was added to the center of the filter membrane. After one minute, the tube was centrifuged at 10,000 x g for 1 minute. The flow through was then checked for DNA concentration and purity by OD_{260} and $OD_{260/280}$ as well as agarose gel electrophoresis.

The PCR amplification and purification was done in triplicate. Two samples were sent to Clemson University Genomics Institute (CUGI, Clemson, SC) along with primers Fred and Renée for sequencing, and one sample was sent to Macrogen (Seoul, Korea). The six resulting sequences were then assembled in Sequencher™ 4.7 from Gene Codes Corporation (Ann Arbor, MI) and edited manually.

The National Center for Biotechnology Information (NCBI) Basic Local

Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to compare the final sequence to previously published sequences using nucleotide-nucleotide (blastn) and translated nucleotide (tblastx) queries. Three of the top matches were used to perform a ClustalW2 alignment from the European Bioinformatics Institute (Cambridgeshire, United Kingdom, software available from <http://www.ebi.ac.uk/Tools/clustalw2/index.html>) to compare identity, strong similarity and similarity of the putative HCCA isomerase to those of other sphingomonads. Only the putative coding region of the nucleotide sequence was used for ClustalW2 alignment.

Sub-cloning *nahD* into *Escherichia coli*

The putative *nahD* gene from EPA 505 was sub-cloned into a pET-30 vector and transformed into a strain of *E. coli* suitable for plasmid maintenance. The plasmid was then extracted and transformed into another strain of *E. coli* suitable for protein expression. Successful transformations were confirmed by growth on selective media and by colony PCR targeting the vector on each side of the cloning site.

Sub-cloning was accomplished using a Novagen® pET-30 Ek/LIC kit (Cat. #69077-3) from EMD Biosciences (Darmstadt, Germany). Host strains *E. coli* NovaBlue competent cells (for plasmid maintenance) and *E. coli* BL21(DE3)pLysS competent cells (for protein expression) were provided with the kit. The pET-30 Ek/LIC vector required the insert to have T4 DNA polymerase specific 5' and 3' extensions for ligation independent cloning (LIC) into the vector.

The pET-30 vector itself carried a kanamycin resistance marker, and pLysS carried a chloramphenicol resistance marker.

PCR primers were designed with 14 bp T4 DNA polymerase specific extensions that would allow single-strand exonuclease activity to create compatible overhangs for ligation independent cloning into the pET-30 vector. Forward primer (5' GAC GAC GAC AAG ATG ACC CGC AAG ATC GAC 3') with T_m of 64.9°C, designated "Ferdinand," targeted the non-coding strand at the start codon, and included a 14 bp extension recognizable by T4 polymerase. Reverse primer (5' GAG GAG AAG CCC GGT CCC TAT TTT TCT AAG 3') with T_m of 61.4°C, designated "Rumpelstiltskin," targeted the coding strand at the stop codon, and included a 14 bp extension for T4 polymerase. Forward primer Ferdinand and reverse primer Rumpelstiltskin were obtained from IDT (Coralville, IA) and diluted in nuclease-free H₂O to 10 μMol. The entire PCR amplified product should be 616 bp based on the sequencing data.

The PCR reaction was assembled with the following: 2 μL isolated EPA 505 DNA template, 2 μL forward primer Ferdinand (10 μMol), 2 μL reverse primer Rumpelstiltskin (10 μMol), 19 μL H₂O and 25 μL GoTaq® Green MasterMix. PCR reaction was carried out in a Genius thermocycler with heated lid. Reaction was as follows: 95°C initial denaturation for 2 minutes; 30 cycles of 95°C for 45 seconds, 58°C for 45 seconds and 72°C for 45 seconds; final elongation at 72°C for 2 minutes, then held at 4°C. The PCR product was then purified using QIAquick® PCR purification kit from QIAGEN (Valencia, CA) following

manufacturer's instructions as outlined above. The eluted DNA was then checked for concentration and purity by OD_{260} and $OD_{260/280}$. Then 5 μ L PCR reaction and 5 μ L purified PCR product were analyzed by agarose gel electrophoresis as described previously.

The purified PCR product was then treated for insertion into the vector by T4 DNA polymerase following the manufacturer's instructions as follows. The following were assembled in a 1.5 mL microfuge tube on ice: 3 μ L (0.2 pmol) purified PCR product, 2 μ L 10X T4 DNA polymerase buffer, 2 μ L of 25 mM dATP, 1 μ L of 100 mM DTT, 11.6 μ L nuclease-free H_2O and 0.4 μ L of 2.5 U/ μ L T4 DNA polymerase for a total reaction volume of 20 μ L. The reaction was incubated at 22°C for 30 minutes, and then the enzyme was inactivated by incubation at 75°C for 20 minutes.

The prepared insert was then annealed to the pET-30 Ek/LIC vector by assembling the following components in a 1.5 mL microfuge tube: 1 μ L pET-30 Ek/LIC vector and 2 μ L treated insert. This was incubated at 22°C for 5 minutes before adding 1 μ L of 25 mM EDTA and incubating for an additional 5 minutes.

The annealing reaction was then transformed into *E. coli* NovaBlue competent cells. The competent cells were removed from -80°C storage and allowed to thaw on ice for 2 minutes. The tube was gently flicked to resuspend the 50 μ L of cells, and then 1 μ L of annealing reaction was added and stirred with a sterile pipet tip. This was incubated on ice for 5 minutes. The tube was then heated for 30 seconds in a 42°C water bath and returned to ice for 2

minutes. After incubation, 250 μ L of room temperature SOC medium was added and incubated at 37°C for 1 hour with 200 rpm in a G24 Environmental Incubator Shaker. Lastly, 50 μ L of transformation culture was plated onto each of two LB plates with 30 μ g/mL kanamycin A from Sigma Chemical (St. Louis, MO) and incubated overnight at 37°C.

Colonies able to grow on LB/kanamycin were checked by colony PCR to verify the presence of vector and insert, ensuring a successful transformation. PCR primers were designed to target sites upstream and downstream of the insert cloning site for amplification. Forward primer (5' TAA TAC GAC TCA CTA TAG GGG 3') with T_m of 50.1°C, designated "T7 promoter," targeted the T7 promoter sequence of non-coding strand 221 bp upstream of the insert cloning site. Reverse primer (5' TGC TAG TTA TTG CTC AGC GG 3') with T_m of 55.0°C, designated "T7 terminator," targeted the T7 terminator sequence of the coding strand 164 bp downstream of the insert cloning site. Forward primer T7 promoter and reverse primer T7 terminator were obtained from IDT (Coralville, IA) and diluted in nuclease-free H₂O to 10 μ Mol. The entire PCR amplified product should be 973 bp if the colony contains pET-30 with the proper insert. The PCR product should be only 385 bp if the colony contains pET-30 without any insert, and no PCR product should be formed if the colony does not contain pET-30.

Six colonies were picked with sterile toothpicks from the transformation plates and touched to a reference plate of LB/kanamycin before transferring each of the colonies to 0.5 mL tubes with 50 μ L sterile water. The cell suspensions were

vortexed thoroughly and boiled for 5 minutes to lyse the cells followed by centrifugation at 10,000 x g for one minute to pellet cell debris. The supernatant was then used as template DNA for colony PCR.

The colony PCR reaction was assembled with the following: 10 μ L supernatant DNA template, 1 μ L forward primer T7 promoter (10 μ Mol), 1 μ L reverse primer T7 terminator (10 μ Mol), 13 μ L H₂O and 25 μ L GoTaq® Green MasterMix. PCR reaction was carried out in a Genius thermocycler with heated lid. The reaction was as follows: 95°C initial denaturation for 2 minutes; 30 cycles of 95°C for 45 seconds, 58°C for 45 seconds and 72°C for 45 seconds; final elongation at 72°C for 2 minutes, then held at 4°C. The PCR reaction was analyzed by agarose gel electrophoresis as described previously to determine PCR product size.

One of the reference colonies that was determined to be a successful transformant was used for long-term storage and all subsequent work. The reference colony of *E. coli* NovaBlue recombinant with pET-30 containing the *nahD* insert was inoculated into 5 mL LB/kanamycin and incubated at 37°C overnight with 200 rpm shaking in a G24 Environmental Incubator Shaker. Once the culture had reached OD₆₀₀ of 0.605, 0.9 mL culture was added to 0.1 mL of 80% glycerol to make an 8% glycerol stock culture that was stored at -80°C.

The NovaBlue recombinant was useful for the initial transformation and for plasmid maintenance, but *nahD* needed to be further sub-cloned into *E coli* BL21(DE3)pLysS to be useful for protein expression. This second sub-cloning

was accomplished by plasmid extraction from recombinant NovaBlue and transformation of the plasmid into BL21(DE3)pLysS. Successful transformation was judged by ability to grow on kanamycin and chloramphenicol, as pET-30 Ek/LIC and pLysS confer resistance to these antibiotics, respectively. Transformants were also verified by colony PCR as before.

NovaBlue recombinants were cultured for plasmid extraction. A streak isolation was performed onto LB with 30 µg/mL kanamycin A from the stock glycerol culture. This was incubated overnight at 37°C. An isolated colony was inoculated into 10 mL LB/kanamycin and cultured for 12 hours until reaching OD₆₀₀ of 0.618. This was then used for plasmid extraction with a Wizard® Plus SV Miniprep DNA Purification System from Promega (Madison, WI) following manufacturer's instructions as follows. Cells from 10 mL culture were harvested by centrifugation at 10,000 x g for 5 minutes. The supernatant was removed, and 250 µL Cell Resuspension Solution was added and pipetted in and out to resuspend cells, and then it was transferred to a 1.5 mL microfuge tube. After complete cell resuspension, 250 µL Cell Lysis Solution was added, and the tube was inverted four times to mix. This was allowed to incubate at room temperature for 5 minutes while cell lysis occurred. Then 10 µL Alkaline Protease Solution was added and mixed by inversion of the tube four times. This was allowed to incubate for an additional 5 minutes. Next, 350 µL Neutralization Solution was added and mixed by inversion of the tube 4 times. The cell debris was then pelleted by centrifugation at 10,000 x g for 10 minutes. Then 850 µL of the

supernatant lysate was transferred to a Spin Column inside a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through was discarded, and 750 μ L Column Wash Solution was added to the Spin Column. This was centrifuged at 10,000 x g for 1 minute, the flow through was discarded, and the wash procedure was repeated with 250 μ L Column Wash Solution. The dry Spin Column was transferred to a clean 1.5 mL tube, and the DNA was eluted by addition of 100 μ L nuclease-free H₂O followed by centrifugation for 1 minute. The concentration and purity of the DNA were checked by OD₂₆₀ and OD_{260/280}. The DNA was also analyzed by agarose gel electrophoresis as described previously. The plasmid should be approximately 6000 bp with the insert.

The purified plasmid was then transformed into BL21(DE3)pLysS competent cells. The competent cells were removed from -80°C storage and allowed to thaw on ice for 2 minutes. The tube was gently flicked to resuspend the 20 μ L of cells, and then 1 μ L of a 1:10 dilution recombinant plasmid (approximately 3-4 ng) was added and stirred with pipet tip. This was incubated on ice for 5 minutes. The tube was then heated for 30 seconds in a 42°C water bath and returned to ice for 2 minutes. After incubation, 250 μ L of room temperature SOC medium was added and incubated at 37°C for 1 hour with 200 rpm in a G24 Environmental Incubator Shaker. Lastly, 50 μ L of transformation culture was plated onto each of two LB plates with 30 μ g/mL kanamycin A and 34 μ g/mL chloramphenicol from Sigma Chemical (St. Louis, MO) and incubated overnight at 37°C.

Three colonies were subjected to colony PCR with the T7 promoter primer

and the T7 terminator primer as outlined above. PCR products were analyzed by agarose gel electrophoresis as described previously. One reference colony judged to be a successful transformant was chosen to be prepared as a stock culture. It was cultured in 5 mL LB with 30 µg/mL kanamycin A and 34 µg/mL chloramphenicol and 0.5% glucose to inhibit transcription of *nahD*. This was incubated at 30°C overnight with 200 rpm shaking in G24 Environmental Incubator Shaker until reaching OD₆₀₀ of 0.938. Then 0.9 mL culture was added to 0.1 mL of 80% glycerol to make an 8% glycerol stock culture which was frozen and stored at -80°C. This recombinant BL21(DE3)pLysS with *nahD* was used in all subsequent work.

The insert was also re-sequenced to verify successful sub-cloning. PCR with the T7 promoter and T7 terminator primers was again performed as described above. The PCR product was purified with the QIAquick® PCR purification kit from Qiagen (Valencia, CA) and sent to CUGI (Clemson, SC) with the forward and reverse primers for sequencing.

Protein expression

Expression of *nahD* in BL21(DE3)pLysS recombinants was performed and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). First, a small scale induction assay was performed to analyze protein expression, then a medium scale culture was induced for protein harvest.

A streak isolation of recombinant BL21(DE3)pLysS was performed onto LB/kanamycin/chloramphenicol and incubated at 30°C overnight. An isolated

colony was inoculated into 5 mL of LB/kanamycin/chloramphenicol and incubated at 30°C overnight with 150 rpm in a Lab-line Orbit Microprocessor Shaker Bath from Thermo Scientific (Waltham, MA). Once the culture had reached OD₆₀₀ of 0.712, 4 mL of the overnight culture was added to a fresh 100 mL of LB/kanamycin/chloramphenicol in a 500 mL erlenmeyer flask and kept at 30°C with 150 rpm shaking. After 3.5 hours of incubation, the culture reached OD₆₀₀ of 0.677. The culture was aseptically separated into two 50 mL cultures, each in a 250 ml flask. The *lac* promoter inducer isopropylthiogalactoside (IPTG) was added to only one flask to a final concentration of 1 mM. Both cultures continued under the same condition for an additional six hours, and 1 mL samples were taken at 0,1, 2, 4, and 6 hours. The samples were tested for OD₆₀₀ and prepared for SDS-PAGE analysis. One mL of culture was centrifuged at 10,000 x g for one minute, and the supernatant discarded. The cell pellet was resuspended in 100 µL phosphate buffer solution (PBS) for a 10X concentration. Then 100 µL sample loading buffer was added, and the samples were boiled for 3 minutes. Each of the samples was then loaded onto two polyacrylamide gels from Bio-Rad (Hercules, CA). The first gel was an 18-well 10% Tris-HCl (cat. #345-0010), and 30 µL of each sample was loaded as well as 7 µL Precision Plus All Blue Standards (cat. #161-0373) and electrophoresed at 200V for one hour. The second gel was a 12-well 4-10% Tris-HCl (cat. #161-1173), and 20 µL of each sample was loaded as well as 10 µL Precision Plus All Blue Standards and electrophoresed at 100V for 1.75 hours. The gels were stained with Coomassie

Blue stain overnight with gentle rocking, then destained with 1% acetic acid and photographed.

A similar procedure was conducted with a mid-size induced culture for harvesting the expressed enzyme HCCA isomerase. A 125 mL flask with 25 mL LB with 30 µg/mL kanamycin A and 34 µg/mL chloramphenicol was inoculated with three colonies of BL21(DE3)pLysS recombinants from a 24 hour streak isolation. The culture was incubated overnight at 30°C with 150 rpm shaking in a Lab-line Orbit Microprocessor Shaker Bath until reaching OD₆₀₀ of 1.251. At this point, the culture was added to 500 mL of fresh medium in a 2 L flask, and incubation continued under the same conditions. After 2.5 hours the OD₆₀₀ was 0.771, and the culture was induced with 1mM IPTG. Four hours after induction the culture was aliquotted into polypropylene, conical bottom centrifuge tubes and centrifuged at 4°C and 3,900 x g for 20 minutes in an Allegra™ X-22R centrifuge with a SX4250 rotor from Beckman Coulter (Fullerton, CA). The supernatant was removed, and the cell pellet was washed by resuspension in one-tenth volume cold PBS followed by centrifugation for an additional 20 minutes at 4°C and 3,900 x g. The supernatant was discarded, and the cell pellet was stored at -20°C.

Protein purification

Since BL21(DE3)pLysS has plasmid encoded lysozyme, the cell culture need only be subjected to a freeze thaw method to ensure lysis. Purification of HCCA isomerase from the lysate was facilitated by its histidine tagged leader peptide.

The fusion recombinant protein made possible by the pET-30 Ek/LIC vector is easily purified by Ni²⁺ affinity chromatography. Analysis of protein purity was determined by SDS-PAGE, and determination of protein concentration was by UV spectrometry.

Cell lysis was induced by the addition of room temperature, autoclaved buffer (20 mM NaH₂PO₄, 500 mM NaCl, pH 7.5) to the frozen cell pellet. Only 20 mL buffer was used for resuspension of the pellet from 500 mL original culture, creating a 25X concentration of lysate. The cell suspension was pipetted in and out and vortexed to thoroughly mix. Filter-sterilized phenylmethylsulfonylfluoride (PMSF) was added to a final concentration of 1 mM to inhibit protease activity. The solution was then incubated on ice for 45 minutes to allow time for the lysozyme to take effect. Incubation continued at 4°C on a rocking platform from Bellco® Biotechnology (Vineland, NM) for an additional 15 minutes. At this point Triton X-100 was added to a final concentration of 1% (v/v), DNase I from Fisher Scientific (Bohemia, NY) to 10,000 Units/mL and RNase from EMD Biosciences (Darmstadt, Germany) to a final concentration of 5 µg/mL. Incubation at 4°C with rocking continued for an additional 10 minutes. The lysate was then centrifuged at 4°C and 3,500 x g for 30 minutes in a Beckman Coulter Allegra™ X-22R centrifuge with SX-4250 rotor to pellet the cell debris. The supernatant was removed by pipetting and filtered through 0.22 µm filter paper from Whatman (Maidstone, UK) to reduce viscosity.

The Ni²⁺ affinity column was prepared by packing a small amount of glass

wool into the bottom of a 3cc polypropylene syringe. Enough HisLink™ resin from Promega (Madison, WI) was added for a 1 mL settled volume. The column was equilibrated with 5 mL binding buffer (100 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.5) which flowed at approximately 1-2 mL/minute. After the binding buffer had completely entered the column, the filtered lysate was added 1-2 mL at a time. A total of 8 mL lysate was added, and it flowed through the column at about 0.2 mL/min. The resin column was then washed with 20 mL of wash buffer (100 mM HEPES, 500 mM NaCl, 20 mM imidazole, pH 7.5) with a flow rate of 1-2 mL/min. Next, 10 mL elution buffer (100 mM HEPES, 500 mM imidazole, pH 7.5) was added with a flow rate of 1-2 mL/min., and 1 mL elution fractions were collected.

The 10 elution fractions were analyzed by SDS-PAGE to determine the purity of HCCA isomerase recombinant protein. From each of the elution fractions, 30 μ L was added to 10 μ L of 4X SDS sample buffer and boiled for 3 minutes. Then all 40 μ L of each of the first 9 samples was loaded into a 10-well, 10% Tris-HCl polyacrylamide gel (cat. #161-1155) from Bio-Rad (Hercules, CA) with 10 μ L Kaleidoscope Prestained Standards (cat. #161-0324, also from Bio-Rad) in the first lane. The gel was run at 150V for 1.5 hours, stained overnight with Coomassie Blue on a rocking platform, then destained with 1% acetic acid and photographed.

Protein concentration was determined by ultraviolet spectrophotometry using the Warburg formula with OD_{260} , OD_{280} and OD_{320} on the BioPhotometer from

Eppendorf (Hamburg, Germany). The spectrophotometer was blanked with elution buffer, and then each of the 10 elution fractions was tested.

Three of the best elution fractions based on SDS-PAGE and spectrometry analysis were then prepared for long-term storage at -20°C. Glycerol was added to a final concentration of 25%, EDTA to a final concentration of 1 mM and PMSF to a final concentration of 0.5 mM.

CHAPTER FOUR

RESULTS

Sequence and analysis of *nahD*

PCR from *S. paucimobilis* EPA505 chromosomal DNA targeting the putative *nahD* was performed three times with primers Fred and Renée. The concentrations and purity of DNA products were 41.3 µg/mL with OD_{260/280} of 1.81, 39.6 µg/mL with OD_{260/280} of 1.82, and 42.4 µg/mL with OD_{260/280} of 1.79 for the three samples. Gel electrophoresis of each of the three samples showed products of slightly less than 1000 bp. Gel electrophoresis is shown in Figure 4.1.

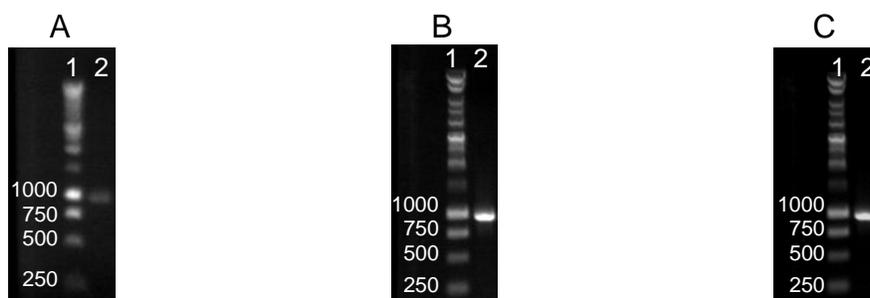


Figure 4.1: Gel electrophoresis of putative *nahD* from *S. paucimobilis* EPA505. Lane 1 of each gel was 6 µL of Promega BenchTop 1kb DNA Ladder with fragment lengths of 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500 and 250 bp. Sizes are marked for the last four bands. Lane 2 of each of the gels was 5 µL PCR sample + 1 µL 6X loading dye.

Sequencing of the PCR product targeting the putative *nahD* from EPA505 resulted in six sequences—each strand sequenced from three reactions. The six sequences were assembled in Sequencher™, and the result is shown in Figure 4.2. The putative start codon ATG and stop codon TAG were identified based on nucleotide and amino acid sequences of HCCA isomerases from other species.

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1   TCCAGCAGATTCAAACACGCTCGCAGTGAGGCAAACCGTGCCCAAGGGT
51  CCCGAAGCGTTCGAAC TGGTTTGGACCCACTTCGGTTACGCCGACGACGA
101 TGCAGAAATGCAGGCAATCCGGCGCAAGCAGACCAACCTTGATCGGTCCG
151 GCCGGGCTGGTTTCGATGGAAGACGGCGAAAGCGGTGGAAATCGTCCAGA
201 ACGCCATTGCCGGCGAGCAGCAGGCAACCTCCTTCATCGCCATGGGGGGG
251 GGCCGCGCCGAAGATGCCGACCATCTTGTACCCGAAGGCGCCATTATCGG
301 CTTC TGGGACAATTACGAGAAGCTCGTCGGCTTCGGGACCGCACGATGAC
351 CCGCAAGATCGACTTCTATTTGACTTCATCAGTCCCTTCAGTACCTCG
401 CGCAGCTGAAGCTCCCTGAAATCGCCCGCAAGGCCGGTTGCGAACTCGAA
451 TATTGGCCGATCGATATCCCCGAGGCGAAGATCGCGCGGGGAAC TACGG
501 CCCGTCCAATCGCGAGGTGGTTCCGAAAATCAAGGTGATGATGGCGGACC
551 TCAACCGGTGGGCCCGCAAATACGACGCTCCCTTGCGTTTTCCGGCCAGC
601 TTTGCCTGCAAGGACTGGAAC TGCGCCACGCTCTATGCGCGCAGCAGGG
651 CAAGGCGGGAGCATTCGTGCGCGCTGCTTACAATCGCATCTGGGGACAGG
701 GGATCGATCCAAGCGACCGCGAAGAACTGCGCGCCTGCGCAGCCGAAGCC
751 GGGCTTGATGCAGAAGCGTTGACTGCCTTCGTGGACTCGTCACTAGGCCA
801 GAACGAATACCGGAAGGTTTCAAGCCAAGCCTATCAGCGGGGAGTCTTCG
851 GCGCGCCATTAATGTTTGTGACGATGAAATCTTCTGGGGCAACGACCGC
901 CTGGAGTTCTTGGAAGAATACTTAGAAAAATAGGGAGAGGAATTTATGTA
951 TAAGAAATCGTCAATCGAAACCGCTAAGC

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Figure 4.2: Sequence of putative *nahD* from *S. paucimobilis* EPA505. The putative start and stop codons of *nahD* are underlined. This sequence represents the assembly of six repetitions of sequencing.

MTRKIDFYFDYFDFISPFYSYLAQLKLPFIARKAGCELEYWPIDIPEAKIAAGN	50
YGPSNREVVVKIKVMMADLNRWARKYDAPLRFPA SFACKDWN CATLYARE	100
QGKAGAFVAAA YNRIWGQGIDPSDREELRACAAEAGLDAEAL TAFVDSSL	150
GQNEYRKVRSQAYQRGVFGAPLMFV DDEIFWGNDRLEFL EEEYLEK	195

Figure 4.3 Translation of *nahD* from *S. paucimobilis* EPA505

The sequence was analyzed by BLAST, and the results indicated strong matches to several previously sequenced HCCA isomerases from other species. The translated nucleotide sequence was then further compared to some of the top BLAST matches. Three were chosen for further comparison by ClustalW2 alignment—*Sphingobium yanoikuyae*, *Sphingomonas chungbukensis* and *Novosphingobium aromaticivorans*. All four species showed 75% identity, 9%

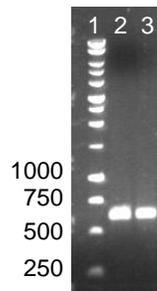


Figure 4.5: Gel electrophoresis for PCR of *nahD* with T4 polymerase extensions. Lane 1 was 6 μL of Promega BenchTop 1kb DNA Ladder with fragment lengths of 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500 and 250 bp. Last four bands labeled. Lane 2 was 5 μL PCR sample + 1 μL 6X loading dye. Lane 3 was 5 μL purified PCR sample + 1 μL 6X loading dye.

After the PCR product was annealed to pET-30 Ek/LIC and transformed into *E. coli* NovaBlue, 171 and 197 colonies grew on the two LB/kanamycin plates after 24 hours. Six of the colonies (three from each plate) were checked for presence of the insert by colony PCR using T7 promoter and T7 terminator primers. Gel electrophoresis of these PCR reactions showed four out of the six reactions to produce bands of lengths close to 1000 bp (lanes 1, 2, 4 and 6). One of the PCR reactions (lane 5) produced a fragment between 250 and 500 bp and another faint band between 500 and 750 bp. One PCR product did not produce any visible bands on the gel (lane 3). The gel electrophoresis is shown in Figure 4.6.

Plasmid was extracted from a successful NovaBlue transformant, and the DNA concentration was found to be 34.8 $\mu\text{g}/\text{mL}$ with $\text{OD}_{260/280}$ of 1.83. Gel electrophoresis of the purified plasmid DNA produced a single band between 5000 and 8000 bp in length. Gel electrophoresis is shown in Figure 4.7.

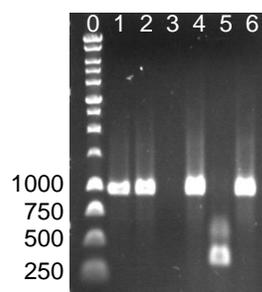


Figure 4.6: Gel electrophoresis of colony PCR from *E. coli* NovaBlue transformation. Lane 0 was 6 μ L of Promega BenchTop 1kb DNA Ladder with fragment lengths of 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500 and 250 bp. Lanes 1-6 were the colony PCR reactions from six different clones.

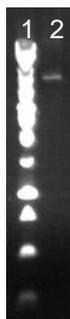


Figure 4.7: Gel electrophoresis of plasmid purification from NovaBlue recombinant. Lane 1 was 6 μ L of Promega BenchTop 1kb DNA Ladder with fragment lengths of 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500 and 250 bp. Lane 2 was 5 μ L isolated plasmid DNA from NovaBlue recombinants + 1 μ L 6X loading dye.

After the plasmid DNA from NovaBlue was transformed into BL21(DE3)pLysS, there were 81 colonies on the first LB/kanamycin/chloramphenicol plate and 24 colonies on the dilution plate. Three colonies were tested by colony PCR using T7 promoter and T7 terminator primers, and all produced DNA fragments close to 1000 bp in length as did most of the PCR reactions from the NovaBlue testing. Gel electrophoresis is shown in Figure 4.8.

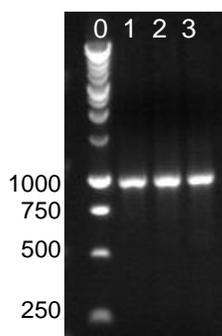


Figure 4.8: Gel electrophoresis of colony PCR for BL21(DE3)pLysS transformation. Lane 0 was 6 μ L of Promega BenchTop 1kb DNA Ladder with fragment lengths of 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500 and 250 bp. Lanes 1-3 were 5 μ L colony PCR products with primers T7 promoter and T7 terminator + 1 μ L 6X loading dye.

Both strands of purified PCR product with T7 promoter and T7 terminator primers were sequenced by CUGI (Clemson, SC). The coding region of the resulting sequence matched perfectly with the previous sequencing results. The complete sequence obtained is shown in Figure 4.9. Approximately 30 bp of sequence was trimmed from each end, since the sequence was poor quality and inconclusive.

Protein expression

During the induction assay for protein expression of HCCA isomerase, the OD_{600} of both IPTG-induced and non-induced culture samples was monitored to ensure similarity in cell density and total-cell-protein quantities. The OD_{600} did, in fact, stay very similar for both cultures as seen in Figure 4.10.

The SDS-PAGE results reveal heavy production of protein shortly after induction only in the induced culture. See Figures 4.11 and 4.12. The expressed protein visible on the gel was estimated to be between 25 and 37 kDa. All other

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001 GGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA
051 TATACATATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCG
101 GTTCTGGTATGAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATG
151 GACAGCCCAGATCTGGGTACCGATGACGACGACAAGATGACCCGCAAGAT
201 CGACTTCTATTTGACTTCATCAGTCCCTTCAGCTACCTCGCGCAGCTGA
251 AGCTCCCTGAAATCGCCCGCAAGGCCGTTGCGAACTCGAATATTGGCCG
301 ATCGATATCCCCGAGGCGAAGATCGCGCGGGGAACTACGGCCCGTCCAA
351 TCGCGAGGTGGTTCCGAAAATCAAGGTGATGATGGCGGACCTCAACCGGT
401 GGGCCCGCAAATACGACGCTCCCTTTCGCTTTTCCGGCCAGCTTTGCCTGC
451 AAGGACTGGAAGTGCGCCACGCTCTATGCGCGGAGCAGGGCAAGGCGGG
501 AGCATTCGTCGCCGCTGCTTACAATCGCATCTGGGGACAGGGGATCGATC
551 CAAGCGACCGGAAGAACTGCGCGCTGCGCAGCCGAAGCCGGGCTTGAT
601 GCAGAAGCGTTGACTGCCTTCGTGGACTCGTCACTAGGCCAGAACGAATA
651 CCGAAGGTTGGAAGCCAAGCCTATCAGCGGGGAGTCTTCGGCGGCCAT
701 TAATGTTTGTGACGATGAAATCTTCTGGGGCAACGACCGCCTGGAGTTC
751 TTGGAAGAATACTTAGAAAAATAGGGACCCGGGCTTCTCCTCAACCATGGC
801 GATATCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGGCGCCGCACTCG
851 AGCACCACCACCACCACCCTGAGATCCGGCTGCTAACAAAAGCCCGAAAG

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Figure 4.9: Sequence of pET-30 recombinant between T7 promoter & T7 terminator. The start and stop codons of the coding region between the T7 promoter and T7 terminator have been underlined.

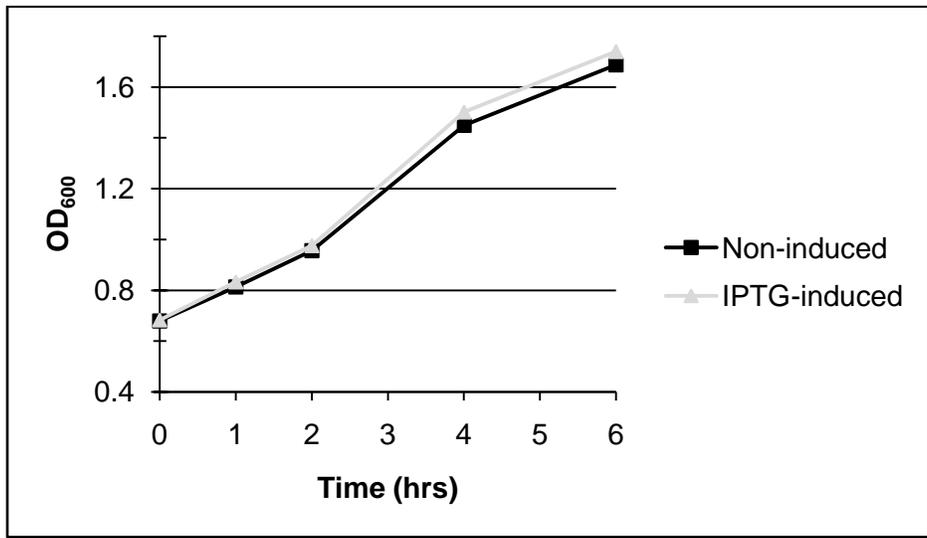


Figure 4.10: Cell density (OD₆₀₀) of cultures for induction assay. Measurements of cell density based on OD₆₀₀ began at induction with IPTG (0 hours). The spectrophotometer was blanked with LB. Samples at 4 and 6 hours were diluted 50% in LB to stay within measureable range, and the OD₆₀₀ values on the chart are double the actual diluted values to reflect the rising cell density trend.

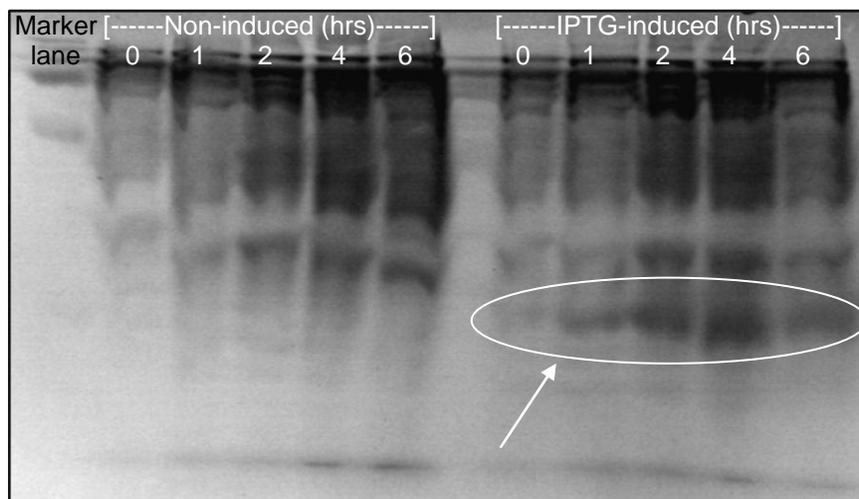


Figure 4.11: SDS-PAGE of IPTG-induced and non-induced BL21(DE3)pLysS. The marker lane was loaded with 7 μ L Bio-Rad Precision Plus All Blue Standards with marker sizes of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. (Most of the marker bands are not clearly visible.) Each of the non-induced and IPTG-induced lanes represents 30 μ L of 10X concentrated sample with loading buffer. The expressed protein circled above was tentatively identified as HCCA isomerase.

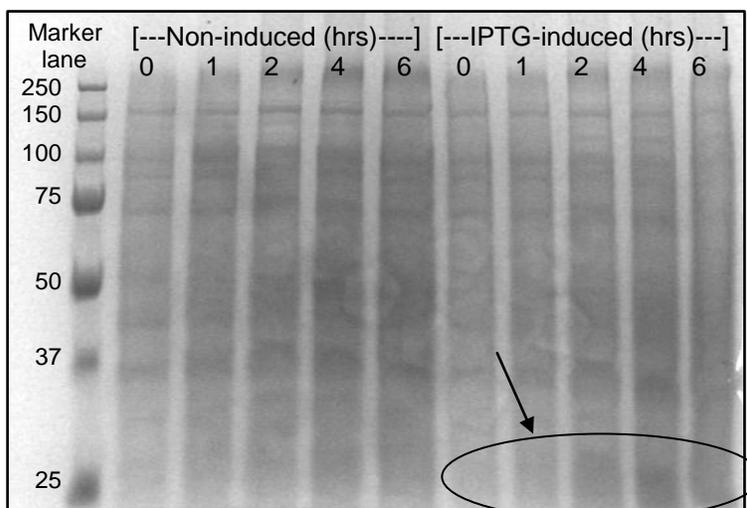


Figure 4.12: SDS-PAGE of IPTG-induced and non-induced BL21(DE3)pLysS. The marker lane was loaded with 10 μ L Bio-Rad Precision Plus All Blue Standards with marker sizes of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Each of the non-induced and IPTG-induced lanes represents 20 μ L of 10X concentrated sample with loading buffer. The expressed protein circled above was tentatively identified as HCCA isomerase.

protein bands appear very similar from both the induced and non-induced cultures. Two gels were run, as the results from only one were not conclusive enough. The first gel (Figure 4.11) clearly showed heavy production of the expressed protein in the IPTG-induced samples beginning one hour after induction and increasing with time, but the marker bands were unclear as they had been washed out with de-stainer. However, enough marker was present to extrapolate the estimated size of the expressed protein to be between 25 and 37 kDa. The second gel (Figure 4.12) had clearly visible marker but only hazy representation of the expressed protein. It was more obvious on the second gel that the expressed protein was closer to the 25 kDa marker than the 37 kDa marker. The best representation of expressed protein on the second gel is seen in the 2 and 4 hour samples from the IPTG-induced culture.

Protein purification

The concentration and purity of histidine tagged HCCA isomerase eluted from the Ni²⁺ resin column was determined by spectrometry and SDS-PAGE. The Eppendorf BioPhotometer measures OD₂₆₀, OD₂₈₀ and OD₃₂₀, and uses the Warburg formula to calculate protein concentration. The results are shown in Figure 4.13. The protein concentration values derived from a Warburg calculation are not exact, but should at least accurately reflect relative concentrations of protein from each elution fraction. The concentrations were highest in elutions 2-6. The SDS-PAGE (Figure 4.14) clearly indicated large amounts of a protein of approximately 25-37 kDa being eluted from the column. Small amounts of

extraneous protein are still present in the first 3 mL eluted, but the remaining elutions appear very pure.

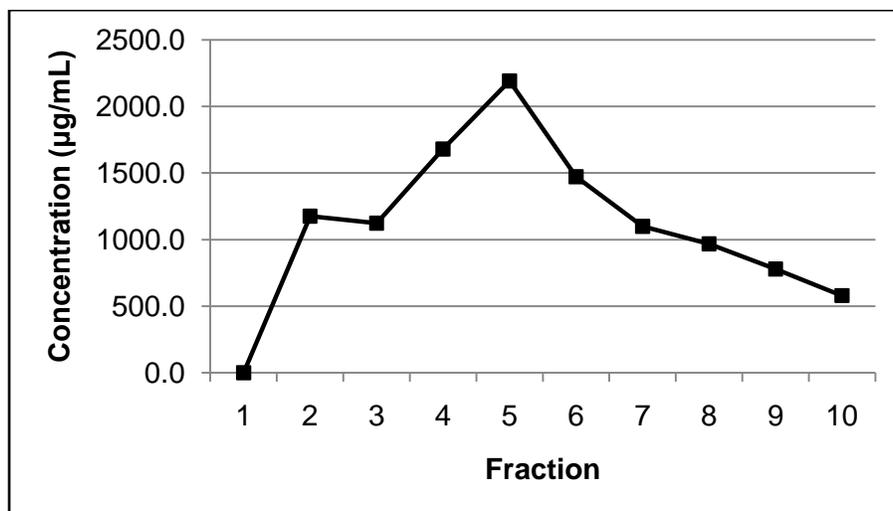


Figure 4.13: Protein concentration of 1 mL fractions eluted from Ni²⁺ resin column. The fractions were 1 mL each. The Biophotometer was calibrated with elution buffer.

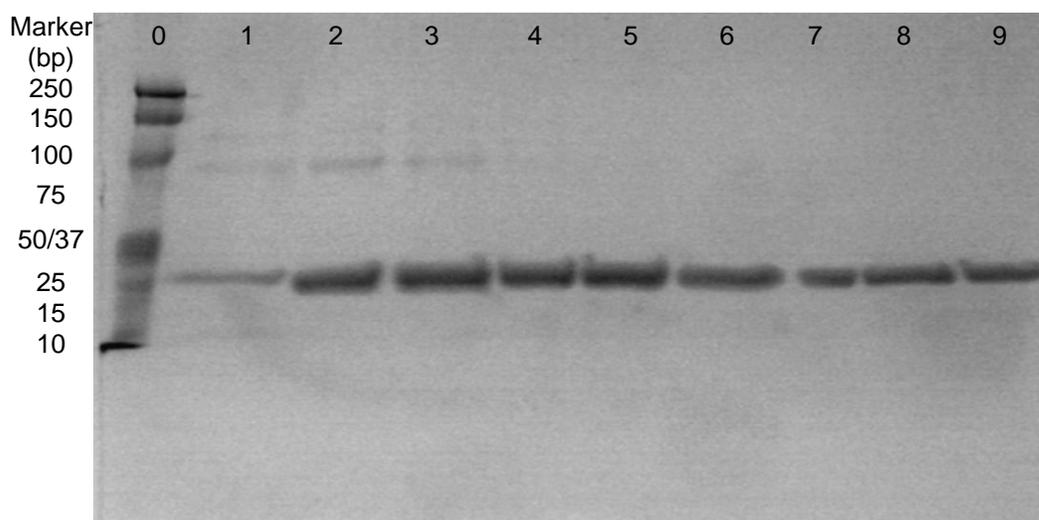


Figure 4.14: SDS-PAGE of eluted fraction from Ni²⁺ resin column. Lane 0 was loaded with 10 µL Bio-Rad Kaleidoscope Prestained Standards with marker sizes of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Each of lanes 1-10 was loaded with 40 µL sample + loading buffer from the corresponding elution fraction.

The most concentrated and most pure fractions were determined to be the 4th, 5th and 6th based on spectrometry and SDS-PAGE. These were the fractions chosen to be stored at -20°C in 25% glycerol, 1 mM EDTA and 0.5 mM PMSF.

CHAPTER FIVE

DISCUSSION

Every line of evidence indicates that the putative *nahD* has been correctly identified and sub-cloned from *S. paucimobilis* EPA505. The PCR amplification from genomic DNA yielded fragments of approximately 1000 bp (see Figure 4.1), as expected. Preliminary sequencing data had indicated a predicted length of 985 bp. The nucleotide sequence obtained was highly similar to previously published sequences belonging to HCCA isomerases from other species as shown by the BLAST queries, and the amino acid sequence is also highly similar to HCCA isomerases from other species as demonstrated by the ClustalW2 alignment (see Figure 4.4). The ClustalW2 alignment showed 75% identity and 17% similarity for the enzyme between EPA505 and three other sphingomonads.

PCR with T4 DNA polymerase extensions for insert of *nahD* into the pET-30 Ek/LIC vector also appears to have been achieved. The PCR reaction should have produced a 616 bp fragment, and the gel in Figure 4.5 shows fragments between 500 and 750 bp. Colony PCR results indicate that *nahD* carried on the pET-30 vector was successfully sub-cloned into *E. coli* NovaBlue and BL21(DE3)pLysS. The primers used targeted the vector on each side of the insert cloning site. If the vector had not been transformed into *E. coli*, then no PCR product would form. This was seen in only one of the six reactions—the fifth colony from the NovaBlue transformation (see Figure 4.6). If the vector had closed on itself and been transformed into *E. coli*, then PCR would produce

fragments of 385 bp in length. This was also seen in one clone from the first NovaBlue transformation (see Figure 4.6). If the vector contained the 588 bp coding region from *nahD*, then PCR would produce fragments of 973 bp in length. This was seen in four of the first six reactions from the NovaBlue transformation (see Figure 4.6) and all three of the reactions from the BL21(DE3)pLysS transformation (see Figure 4.8). The plasmid also appears to have been isolated from NovaBlue for transformation into BL21(DE3)pLysS. The gel electrophoresis of isolated plasmid DNA showed a band corresponding to between 5000 and 8000 bp, and the pET-30 vector with the insert should be approximately 6000 bp. The insert region of the vector was re-sequenced in the final sub-clone, and the results completely matched the previous sequencing results (see Figures 4.2 and 4.9), showing that the integrity of DNA sequence had not been compromised in the cloning process.

Expression and purification of HCCA isomerase also appears to be successful. Before the SDS-PAGE of an induction assay, the protein quantities loaded into the gel must be standardized to ensure a proper comparison. This was achieved by monitoring the cell densities of the induced and non-induced cultures. Since the OD_{600} of both cultures remained nearly identical (see Figure 4.10), then the protein sample load in the SDS-PAGE should also be comparable. The SDS-PAGE from the induction assay clearly showed expression of an approximately 25-37 kDa protein only after induction with IPTG (see Figures 4.11 and 4.12). The recombinant HCCA isomerase with fused

leader peptide was expected to be 27 kDa—22.2 kDa for the HCCA isomerase itself and 4.8 kDa for the vector coded leader peptide. Since the HCCA isomerase should be heavily transcribed after induction with IPTG, it was concluded that the dark protein bands circled in Figures 4.11 and 4.12 correspond to this enzyme. This same 27 kDa protein is seen isolated after SDS-PAGE of the Ni²⁺ affinity column purification (see Figure 4.14), indicating successful purification of the HCCA isomerase enzyme.

These results strongly suggest that HCCA isomerase has been correctly identified, sequenced, sub-cloned, expressed and purified. The enzyme is most likely an integral part of PAH catabolism in *S. paucimobilis* EPA505 since it has been demonstrated to be essential in other species^[19]. Further work should address the catalytic properties of the purified enzyme. The leader peptide could easily be cleaved by reaction with the peptidase enterokinase, leaving HCCA isomerase in its native form. The enzyme could then be reacted with its substrate 2-hydroxychromene-2-carboxylate and with the supposed cofactor glutathione to determine if the expected product *trans*-*o*-hydroxybenzylidenepyruvate is indeed produced. Results of such a study could be conclusive but are not easily accomplished. The substrate is not commercially available or easily synthesized. Previous studies that accomplished the proposed assay did so by one of two methods. In the first method, 2-hydroxychromene-2-carboxylate was accumulated and purified from a culture containing knock-out mutants of the gene *nahD*. In the second method, both HCCA isomerase and the previous

enzyme of the naphthalene catabolic pathway 1,2-dihydroxynaphthalene dioxygenase were purified and reacted together with the previous substrate in the pathway 1,2-dihydroxynaphthalene, which is commercially available.

Further studies should also include the other enzymes of the naphthalene catabolic pathway. There are six enzymes in the upper catabolic pathway. One has been successfully sub-cloned from EPA505 in this study, and two others are currently being researched. If all six enzymes were sub-cloned, expressed and purified, then the entire metabolic pathway could be demonstrated *in vitro*. Genetic organization and regulation of the metabolic pathway should also be researched. Large segments of plasmids or chromosomes coding for PAH catabolic genes have been published for a few species, but little understanding of the regulation and expression of these genes is available.

The results of this study are one step in the progress to fully understand the polycyclic aromatic hydrocarbon metabolism of sphingomonads, but such an understanding is necessary if the goal of bioremediation is to be achieved. The cost benefits could be great and the environmental impacts also greatly improved if bioremediation ever replaces costly traditional methods of remediation. Such ambitions should be pursued as environmental stewardship of this planet is in human hands.

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