THE SCREENING OF INTESTINAL BACTERIA FOR THE PRESENCE OF CYTOCHROME P450 WITH INDUCTION BY PHENOBARBITAL

By

SHANNON DALE WALLS

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1995

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2001

THE SCREENING OF INTESTINAL BACTERIA

FOR THE PRESENCE OF CYTOCHROME

P450 WITH INDUCTION BY

PHENOBARBITAL

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

PREFACE

We know that cytochrome P450 plays a role in the toxification and/or detoxification of many xenobiotics. We also know that P450 is found in many organisms including bacteria and man. So when man is exposed to a certain xenobiotic (e.g. food dye), bacteria in the intestine are also exposed to the xenobiotic. The purpose of this study was to determine the role of intestinal bacteria in the detoxification of xenobiotics in the intestine. Major strains of intestinal bacteria were screened for the presence of cytochrome P450, which is not known to exist currently in intestinal bacteria. The results of this study will contribute towards our understanding of how intestinal bacteria respond to xenobiotics, and to determine the role intestinal bacteria play in xenobiotic metabolism as it relates to cancer and bioremediation research.

I would like to thank my mom for being a sounding board. I would also like to thank Jennifer, who kept pushing me to finish. I would like to thank my major advisor Dr. Gilbert John for giving me a project that allows me to continue to enhance my understanding of science. And finally I would like to extend my gratitude to my committee, Dr. Jim Blankemyer and Dr. Ulrich Melcher.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

Introduction and Literature Review

History of Cytochrome P450

For many years a war has been raging between plants and animals. When animals first began crawling out of the ocean in search of new food sources, they encountered land plants. Initially, land plants were not affected, but, as time passed, these animals began to ingest plants. As a defense mechanism, plants produced stress metabolites called phytoalexins, which made plants less palatable and/or digestible. In turn, animals responded with the production of an enzyme system called cytochrome P450, which detoxified these phytoalexins ([1]).

Cytochrome P450 may have evolved from bacteria millions of years ago. Bacterial evolution pre-dates drugs, animal-plant warfare, and the combustion of organic matter. Therefore, it is possible that P450 may have played a role in the oxidation of the atmosphere (i.e. the reduction of Nitrogen). Cytochrome P450 may also have played a role in oxygen-detoxification during the chemical evolution period, as P450 may have been involved in converting H_2O_2 to H_2O ([2], [3], and [4]).

In the early 1950's, a new type of cytochrome found in rat liver microsomes was observed ([5]). A broad absorption band with a maximum absorption at 450 nm detected after treating the sample with a reducing agent

and carbon monoxide (CO). This absorption band was later called the CO difference absorption spectrum.

Later, other investigators confirmed the presence of the absorbance spectrum using microsomes isolated from pig liver ([6]). In addition, these studies determined that the reducing agent dithionite had a similar redox potential as cysteine, thereby, suggesting that the protein(s) in pig liver microsomes contained a metal ion ([6]).

In the early 1960's, the term P-450 was coined ([7]). During this time investigators determined that cytochrome P450 was not related to other known cytochromes such as cytochrome b_5. In addition, investigators determined that the P-450 protein was a hemoprotein. Further characterization of cytochrome P450 revealed the denatured form of the protein produced an unusual characteristic absorption peak at 420 nm ([7]). In addition, it was determined that cytochrome P450 had characteristics of a *b*-type cytochrome ([8]).

In 1968, the first bacterial P450 from *Pseudomonas putida* (P450CAM) was isolated ([9]). The P450CAM was confirmed as a b-type cytochrome with an iron protoporphyrin IX-containing protein. In 1985, P450CAM was the first three-dimensional crystal structure of cytochrome P450 to be determined ([10]). The crystal structure was later used as a model to provide information on the structure and function of bacterial and mammalian P450s.

Characteristics of Cytochrome P450

The unique spectral property of all cytochrome P450s is the production of the 450 nm spectral peak. The 450 nm peak is due in part to the heme-iron binding to the sulfur atom on the amino acid cysteine. Specifically, the fifth ligand of the heme binds to cysteine, while CO binds at the sixth ligand on the opposite side of the heme ([11]). The cysteine residue is near several highly conserved proteins, thereby, making up the largest conserved region of the protein. In addition, other minor conserved regions exist.

Most all known bacterial cytochrome P450s consist of a two-protein system, except for *Bacillus megaterium*. *B. megaterium* has three distinct P450 systems. One of the three P450 proteins in *B. megaterium* called BM-3 has a one-protein system, in which the FAD, FMN, and a P450 heme moiety make-up the large 119 kDa protein. The protein is self-sufficient as the protein combines both the electron transport functions and substrate oxygenation function in one large molecule. BM-3 was first detected as a fatty acid hydroxylase in cell-free preparations and later identified as a P450 ([12]). BM-3 is inducible by phenobarbital, and is one of only two bacterial P450 systems (the other being from *Streptomyces griseolus* ([13])) that are inducible by phenobarbital (a xenobiotic which is a man-made or foreign compound). The other two P450 proteins, BM-1 and BM-2, have a two-protein system. BM-1 has a molecular weight near 47kDa and resembles P450CAM from *P. putida* ([12]). BM-2 has a molecular weight of 48 kDa and is poorly characterized, as the protein has not been cloned intact.

Cytochrome P450s are classified as a super-family of heme proteins. P450s are divided into families and sub-families based on amino acid sequence identity. If the amino acid sequence is >40% identical to an existing family, then that P450 sequence lies within the same family. If a P450 protein sequence from one gene family is $\leq$ 40% identical to an existing family, it will be placed in a separate family ([14]). A sub-family is a branch of a family in which more than one P450 enzyme exists. A cytochrome P450 gene is designated as *CYP* (from cytochrome P450). Different cytochrome P450s are distinguished by Arabic number and letter designations. For example, CYP3A4, the Arabic number denotes the family, the letter designates the subfamily, and second Arabic number represents the individual gene within the subfamily.

An interesting aspect of the cytochrome P450 protein includes a signature amino acid sequence FxxGxxxCxG, where the cysteine of the CxG refers to the enzyme active-cysteine ([14]). There are no prokaryotic sequences that deviate from the original signature sequence, however a few eukaryotic sequences have slight deviations. Currently, there are 74 gene families and within that number, only 14 families (which include 26 sub-families) are mammalian associated ([14]).

There are three general types of compounds that are known to induce the different cytochrome P450 systems: 1) drugs, 2) toxic or carcinogenic compounds and 3) steroids. Most cytochrome P450 systems are inducible, but the mechanism of the induction is not well understood. What is known is that the induction of P450 into bacterial systems can be classified into three categories: 1) substrate induction and substrate analogs, 2) non-substrate induction by

exogenous or endogenous substances and, 3) induction by environmental factors ([12]). In addition, P450 induction by phenobarbital is said to be a regulated process in mammals, avian species, and in bacteria ([15]). In mammals, induction with phenobarbital causes a 50- to 100-fold increase in the P450 protein, mRNA, and transcription initiation. In bacteria, the activation of transcription for P450BM-3 is under positive control (presence of inducer), as a transacting factor protein interacts with the 5'-flanking region of the *BM*-3 gene. Based on similar sequence identity at the 5'-flanking region for both mammals and bacteria, the regulatory mechanisms associated with phenobarbital induction may be similar for both systems.

Figure 1 shows the three cytochrome P450 protein systems. The three systems are based on the minimum number of protein components necessary to reconstitute monooxygenase activity ([12]).

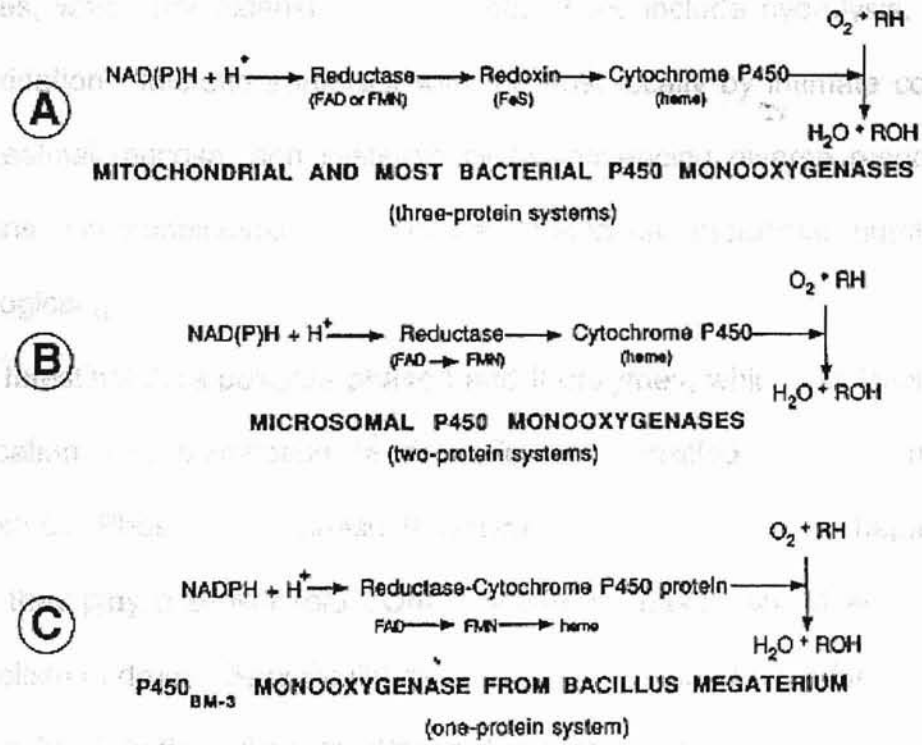

Fig. 1. Cytochrome P450 protein systems. Comparisons of mitochondrial, microsomal, and bacterial P450 enzyme systems ([12]).

Recently, a fourth type of P450 was identified, P450nor. P450nor is unique in that it receives its electrons directly from reduced pyridine nucleotides without the intervention of an electron carrier (reductase or redoxin) ([16]).

Intestinal Microflora

Different specific intestinal bacterial (microflora) populations exist in humans depending on diet, but, in general, the various kinds of bacterial species present in the intestine are the same for all individuals. There have been more than 400 bacterial species identified from feces, with anaerobic bacteria being the predominant type of microorganism ([17], [18], [19], and [20]). The diversity and abundance of bacteria in the gut are reflected in their collective metabolic

activities, which are extensive and varied. They include hydrolysis, reduction, and oxidation. Microflora interact with the host locally by intimate contact with the intestinal mucosa, and systemically by influencing diverse responses and functions – immunological, physiological, anatomical, metabolic, nutritional, and toxicological ([21]).

Intestinal flora possess phase I and II enzymes, which are involved in the modification and elimination of detoxified and toxified products from living organisms. Phase I and phase II enzymes are also found in hepatic tissue, where they play a similar role. One function of phase I and II enzymes is the metabolism of drugs. Specifically, phase I enzymes introduce a functional group, such as OH, into the substrate. Phase II enzymes use this functional group as a "handle" for conjugation, yielding a hydrophilic product, thereby, promoting the excretion of the generated product ([1], [22]).

The microflora and liver are believed to function together through a process called hepatic recycling. The intake of some oral medications are initially metabolized by microflora using phase I and II enzymes. Some of these products may absorb into the intestine and make their way to the liver. The phase I and II enzymes from the liver will further metabolize the compound. After passage through the liver, the compounds that are less than 56 kDa are excreted into the kidneys where they are eliminated as urine. Larger molecules re-enter the intestine via the bile (hepatic cycling) and are eliminated via feces. In some instances the compound or its metabolites may be re-absorbed and circulated back to the liver ([22]).

Since the liver and intestinal flora both possess phase I and II enzyme systems, certain enzyme reactions in the liver are similar to those in bacterial flora. For example, azoreductase is a phase I enzyme, that is present in mammals and bacteria. Azoreductase reduce azo dyes to amines, which can be mutagenic and carcinogenic. Specifically, the azoreductase in mammalian liver has been identified as cytochrome P450. Unfortunately, the structure and function of bacterial azo-reductase is not fully characterized ([23]). Therefore, bacterial azo-reductase maybe a cytochrome P450 protein. In addition, there has not been an extensive study to determine the presence of cytochrome P450 in intestinal microflora.

Due to the diverse metabolic capacity of intestinal microflora, and the lack of information regarding the specific enzymes present in specific species of bacteria, the proposed study seeks to use a number of molecular biology, biochemistry and microbiology techniques to investigate the presence of cytochrome P450 in intestinal microflora.

CHAPTER II

PCR amplification to screen for P450

Introduction

Cytochrome P450 may have evolved from bacteria millions of years ago. Therefore, a number of conserved regions may exist between mammalian and bacterial systems, as well as, among the many diverse species of bacteria. This study used a number of cytochrome P450 conserved regions in bacteria to screen other species of bacteria for the presence of P450. Based on this approach, several methodologies for the screening of various intestinal bacteria (Table 1) were developed to screen for the presence of cytochrome P450. First, specific primers were designed based on conserved amino acid regions in P450 from *Pseudomonas putida* (P450CAM) and *Bacillus megaterium* (P450BM-3). The primers were used to PCR amplify the DNA of 12 different bacterial species (Table 1).

SPECIES NAME	ATCC #
Bacteroides stercoris	43183
Bifidobacterium adolescentis	15703
Bifidobacterium bifidum	15696
Clostridium perfringens	19574
Enterococcus faecalis	27274
Enterococcus faecium	6569
Escherichia coli	11698
Eubacterium aerofaciens	25986
Fusobacterium prousnitzii	27768
Ruminococcus bromii	27255
Staphylococcus aureus	13150
Streptococcus intestinalis	43492

Table 1: Bacterial species studied using PCR.
All strains were obtained from American Type Culture Collection (ATCC).

Second, different DNA sample preparation methods were tested using the PCR technique. They included: 1) whole cell, 2) chromosomal, and 3) cDNA preparations. Third, two methods of PCR product analysis were tested. They included 1) electro-elution and 2) the TA vector method. The efficiency and accuracy of the methods was tested by DNA sequence determination.

The methods developed were subsequently used through out the study to screen intestinal flora for the presence of cytochrome P450.

Methods and Materials

Whole cell PCR: Lyophilized whole cells were used to PCR amplified DNA. Twelve ATCC strains were tested (Table 1). The lyophilized cells were first re-suspended in 1 ml ddH_2O. The PCR reaction mixture contained: 10 µl amount of 10X PCR buffer-minus Mg^{2+}(Promega, Madison, WI), 6 µl 0.25 mM $MgCl_2$, 2 µl 10mM dNTPs, 1 µg of primer (Table 2), 1 ml TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), 5 µl whole cell suspension, 0.5 µl *Taq* polymerase (2.5 units) and the volume was brought up to 100 µl using ddH_2O. The mixture was centrifuged briefly (5 seconds) and overlaid with 60 µl mineral oil.

PCR conditions: PCR amplification was performed in a Perkin Elmer Cetus Thermo Cycler (Norwalk, CT). The following cycles were used: One cycle at 94°C for five minutes, 55°C for five minutes, and 72°C for five minutes. These were followed by 30 cycles using the same temperature, but the incubation times were changed to one minute all temperatures. An extension step was performed at the end of the 30 cycles in which the sample was incubated at 72°C for five minutes. The final step was a 4°C soak.

Large-scale preparation of bacterial genomic DNA: A 100 ml sample of bacterial culture was grown overnight and subsequently centrifuged in a Sorvall centrifuge (GSA-13 rotor, Newtown, CT) for 10 minutes at 4000 rpm at 4°C. The pellet was re-suspended in 9.5 ml TE, 0.5 ml 10% SDS and 50 µl of 20 mg/ml proteinase K, vortexed, and incubated for one hour at 37°C. A 1.8 ml amount of 5M NaCl was added and vortexed. Then, a 1.5 ml CTAB/NaCl solution was

added, vortexed again and incubated at 65°C for 20 minutes. An equal volume of chloroform-isoamyl alcohol (24:1) was added, vortexed and centrifuged for 10 minutes at 6000 rpm at room temperature. The top layer was transferred to a fresh micro-centrifuge tube and an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) was added. The sample was inverted 5 times and centrifuged (Eppendorf model 5415c) at 14,000 rpm for 10 minutes. The top layer was transferred to a fresh micro-centrifuge tube. A 0.6 volume of isopropanol was added in order to precipitate the DNA. The sample was centrifuged for 10 minutes at 14,000 rpm, at room temperature. The supernatant was removed and the precipitate was washed with 70% ethanol (EtOH), air-dried and re-suspended in a 100 μl amount of TE buffer. Approximately, 2μg of chromosomal DNA was used in the PCR reactions.

mRNA isolation: The Micro-FastTrack Kit from Invitrogen (Carlsbad, CA) was used to isolate mRNA. Briefly, the bacterial cells were washed and re-suspended in a lysis buffer. Cellulose was used to bind nucleic acid, allowing mRNA to be isolated from the column. The mRNA was then washed from the cellulose.

cDNA synthesis: The 3' RACE kit from Gibco BRL (Rockville, MD) was used to synthesize the cDNA. The mRNA served as the template for the AP primer binding. Figure 2 demonstrates the procedure used to synthesize and test the cDNA, using specific primers. Reverse transcriptase allowed the synthesis of the cDNA from mRNA. The cDNA then served as the template for the binding of specific primers (Table 2).

PCR electrophoresis: A 2% agarose gel (Sigma, St. Louis MO) in TAE (0.04 M tris-acetate and 0.001 M EDTA) was used to analyze all PCR products.

TA vector method: The TA cloning kit from Invitrogen (Carlsbad, CA) was used in ligating the PCR product.

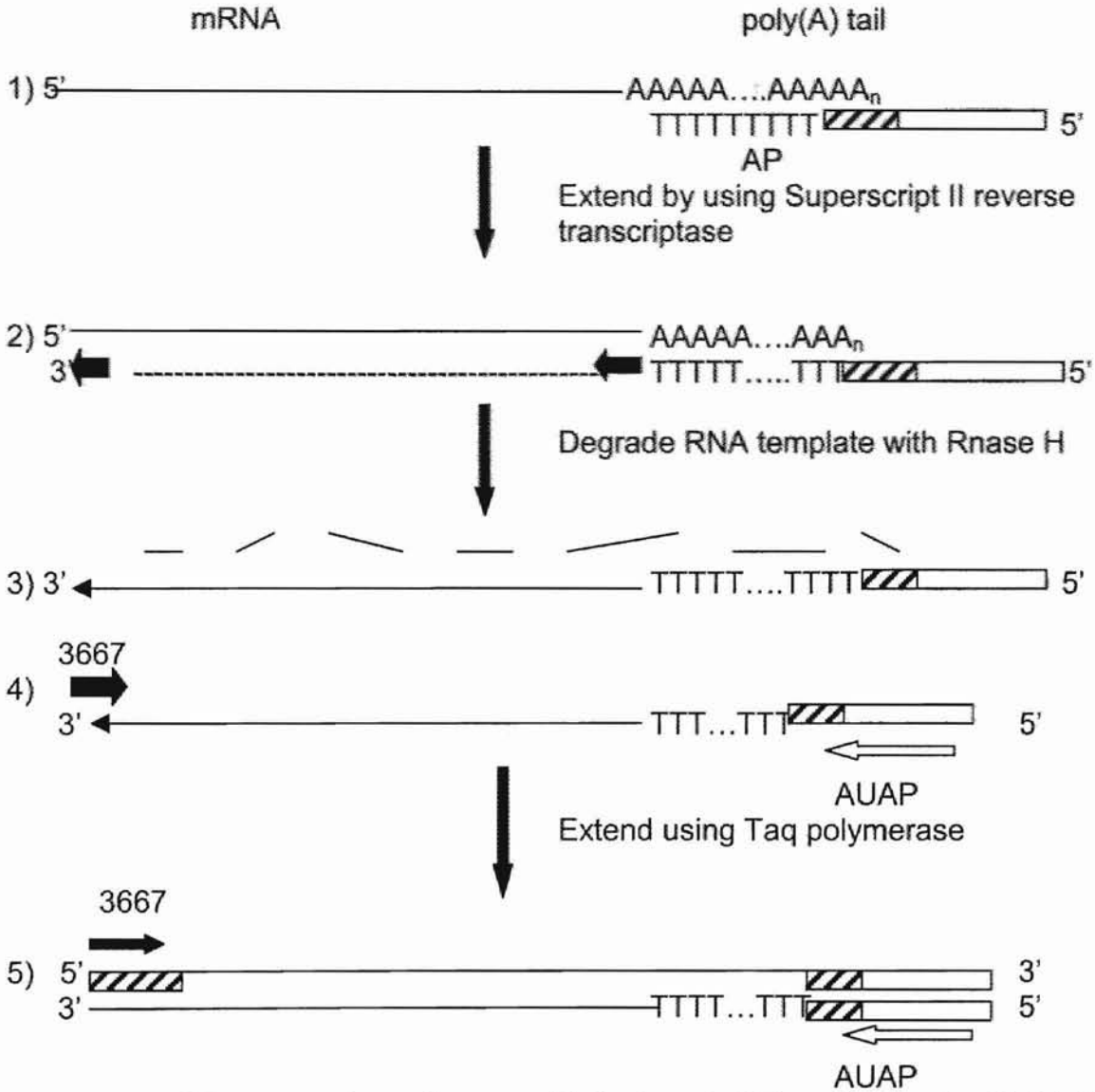

Fig. 2: RT-PCR was performed on a mRNA strand of *E. coli* using the 3' RACE kit from Gibco BRL. AP is an amplified primer that has oligo T and *Mlu* I, *Spe* I, *Sal* I sites and a half *Not* I site. 3667 is a specific primer from *P. putida* and AUAP is the Abridged Universal amplification primer from the Gibco BRL 3' RACE kit.

Electro-elution method:, The agarose gel containing the DNA band was cut out and placed in dialysis tubing (12-14 kDa). Approximately 300 µl TAE buffer was added to the dialysis tubing. The dialysis tubing, which contained the gel was sealed and placed in an electrophoresis unit. The unit was set for 30 minutes at 80 volts. After the electrophoresis, a 10-second reverse pulse (reverse positive and negative wires) was performed. The TAE/DNA mixture was removed from the dialysis tubing and placed in a micro-centrifuge tube. One-half the volume of 7.5M ammonium-acetate was added with 2 times the volume of 95% EtOH. The tube was vortexed and placed on ice for 10 minutes. The suspension was then centrifuged (Eppendorf model 5415c) at 14,000 rpm for 5 minutes at room temperature. The supernatant was removed and the DNA-pellet was rinsed with 80% EtOH and centrifuged again at 14,000 rpm for 2 minutes at room temperature. The supernatant was removed and the pellet was air-dried. The pellet was re-suspended in 75 µl TE buffer.

Nucleotide sequencing: All sequencing was performed at the Recombinant DNA/Protein Resource Facility at Oklahoma State University (OSU).

Plasmid extraction: The Qiagen Plasmid Midi kit (Valencia, CA) was used for the extraction of TA plasmids.

Basic Local Alignment Search Tool (BLAST) ([23]): The BLAST program was used to analyze the sequence information. BLASTP compared an amino acid query sequence against a protein sequence database. BLASTN compared

a nucleotide query sequence against a nucleotide sequence database. BLASTX compared a nucleotide query sequence, translated in all reading frames against a protein sequence database. TBLASTN compared a protein query sequence against a nucleotide sequence database, dynamically translated in all reading frames. The output from each BLAST search had two critical sets of data: 1) an expected value (e-value), and 2) the score number. The e-value was a parameter that described the number of random hits one could expect to see when searching a database of a particular size. The e-value decreased exponentially with the score number. The score number was assigned to a match between two sequences.

Primer Number	Nucleotide Sequence
2533	GCCAAGGCACAGATGGCTGCCGTGGCCAAA
2534	TACTCCGGCGCTTCTCGCTGGTTGCCGATG
3576	CTGGTCGGCGGCCTGGATACG
3667	TTTGGCCACGGCAGCCATCTGTGCCTTGGC
2676	CATATCAAAAGCTGGTGGAAT
2677	TTTGACTTGTTTGTAGCTTGGAA
2678	TCCATAAAAAGCTGGTGCGTATGC
2679	GGCAAGAGGGGCGCCCAAACAAAAATG
2680	TTAAGTCTGAGATAATATCATCAGAAA

Table 2. Nucleotide sequence of PCR primers used. 2533, 2534, and 3576 were specific primers for *P. putida*. 3667 was used for RT-PCR and was specific for *P. putida* P450cam. 2676 and 2677 were specific primers for BM-1. 2678, 2679, and 2680 were specific primers for BM-3.

Results

The approach used in the study was based on previous studies in which the assumption was made that consensus P450 sequences (conserved regions) from one or more species, representing one or more P450 subfamilies, would be represented in the unknown P450 being targeted for cloning ([24]). To test for existing P450 gene sequences in intestinal microflora, the two most extensively studied bacterial P450 systems, P450CAM and BM-3 and BM-1, were used to synthesize several primers specific for the conserved regions. A reverse nucleotide primer was designed based on the universally conserved amino acid cysteine region (FxxGxxxCxG) from *P. putida*. Two forward nucleotide primers were designed based on other minor conserved regions. In addition, two forward and two reverse nucleotide primers were constructed, based on conserved and overlapping amino acid regions from *B. megaterium*.

To determine the best method to use to screen bacteria for the presence of cytochrome P450, several DNA and PCR preparation methods were tested. Initial testing involved *P. putida* P450CAM (Fig. 3) and *B. megaterium* BM-1 and BM-3 (Fig. 4 and 5), as they were used as positive controls. First, chromosomal DNA from *P. putida* was used to test the primers 2534 and 2533. The results produced a predicted size fragment of 200 bp (Table 3). The first method tested for the isolation of PCR fragment and sequencing was the electro-elution method. All PCR products were sequenced at the Recombinant DNA/Protein Resource Facility at OSU. The PCR-DNA sequence was compared to the

nucleotide sequence for P450CAM using the DNASIS program. The PCR-DNA sequence matched 94% for the entire length of *P. putida* P450CAM. Mismatches were seen at the beginning and end of the sequence, which is typically observed with this sequencing approach.

Primer Pair	Predicted size fragment	Species
2533-2534	200 bp	*P. putida*
2533-3576	350 bp	*P. putida*
2676-2677	1000 bp	*B. megaterium*
2678-2679	800 bp	*B. megaterium*
2678-2680	1200 bp	*B. megaterium*

Table 3. Primer number, predicted size fragments for each primer pair and specific species.

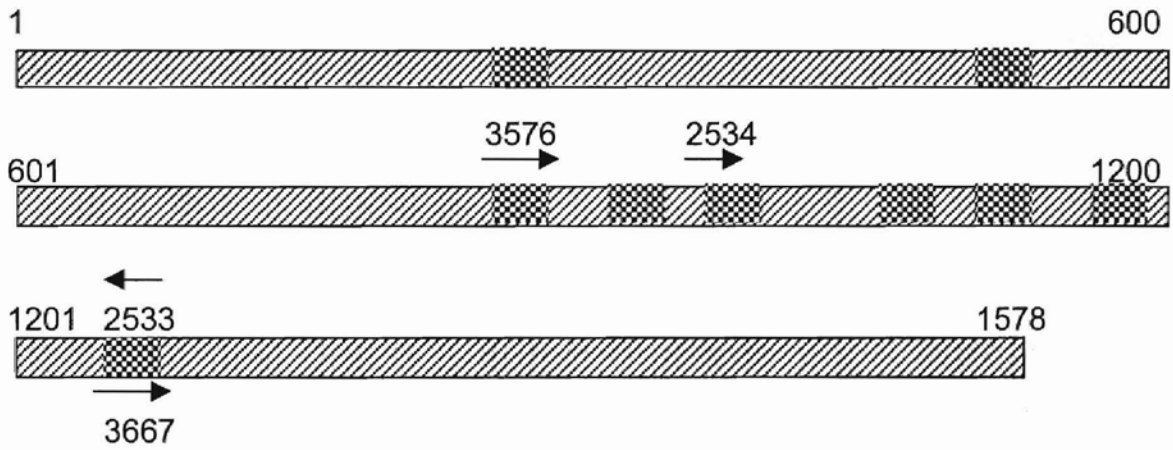

Fig. 3: Representation of the *Pseudomonas putida CAM* gene and its corresponding primers. Primers 3576 and 2534 are specific upstream (forward) primers. Primer 2533 is a specific downstream (reverse) primer and represents the conserved cysteine region. Primer 3667 is a specific primer used in the RT-PCR method (Shaded areas represent conserved regions).

The primers identified in Fig. 4 and Fig. 5 were used to amplify P450 from the control plasmid (pUC13 containing BM-3), a gift from Dr. Fulco (The University of California-Los Angeles) and from *B. megaterium* chromosomal DNA. The predicted size fragments for all primer combinations were generated. They included a 900 bp (2678/2680 primer combination), 1.2 kbp (2678/2679 primer combination), and 1.1 kbp (2676/2677 primer combination) fragment

(Table 3). The sequence information was used to perform a BLASTN search. The search revealed the PCR-DNA sequence had a 95% homology to its corresponding BM cytochrome P450 gene (BM-3 and BM-1).

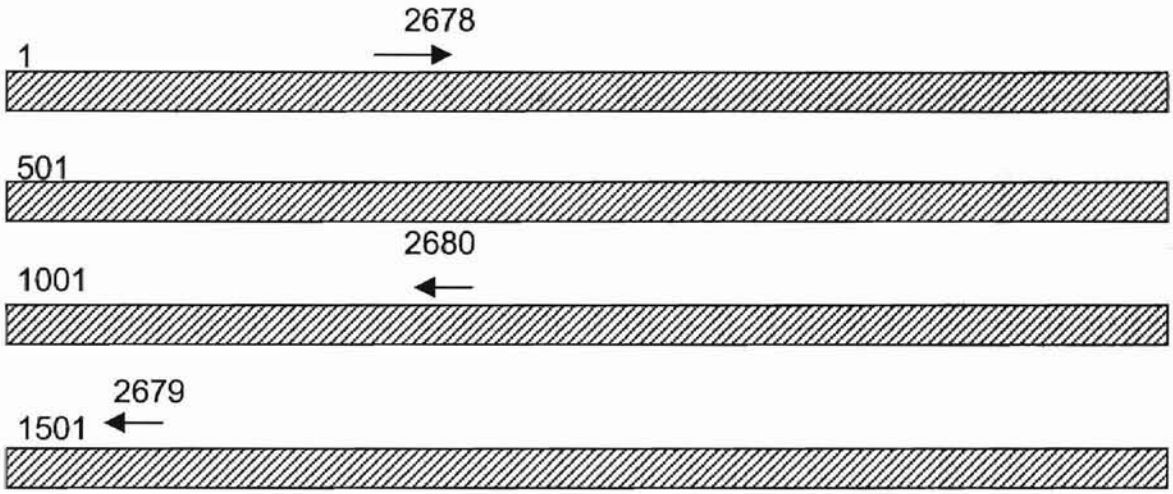

Fig. 4: Representation of the BM-1 gene from *B. megaterium*. 2678 is a specific upstream primer. 2679 and 2680 are specific downstream primers.

To maximize the efficiency of testing twelve intestinal microbes, the whole cell PCR approach was used. Of the twelve bacterial strains tested (whole cell) only *E. coli* produced a 250 bp fragment, which was near the predicted size region of 200 bp. *E. coli* was further investigated as the chromosomal DNA was obtained. Using primers 2533 and 3576, which produces a predicted size fragment of 350 bp, the genomic DNA was PCR amplified. The genomic DNA produced an 800 bp fragment, which was not the predicted size product. Due to the intensity of the 800 bp fragment and the possibility of it containing a P450 sequence, the PCR fragment was isolated by electro-elution and sequenced at the Recombinant DNA/Protein Resource Facility at OSU.

The sequence was analyzed directly by BLASTN and BLASTX. The BLASTN resulted in a score of 418 with 829 bits and an e-value of 0, which is translated as a 94% homology to section 189 of 400 of the *E. coli* chromosome. The BLASTX resulted in a score of 752 with 297 bits and an e-value of 6e-82. The identity was 83% to the putative nucleoside permease protein, from *E. coli*. To determine if section 189 was, in fact, the putative nucleoside permease protein a TBLASTN was performed. The TBLASTN of the putative nucleoside permease protein resulted in a score of 913 with 360 bits and an e-value of e-100, which was 99% identical for section 189 of 400.

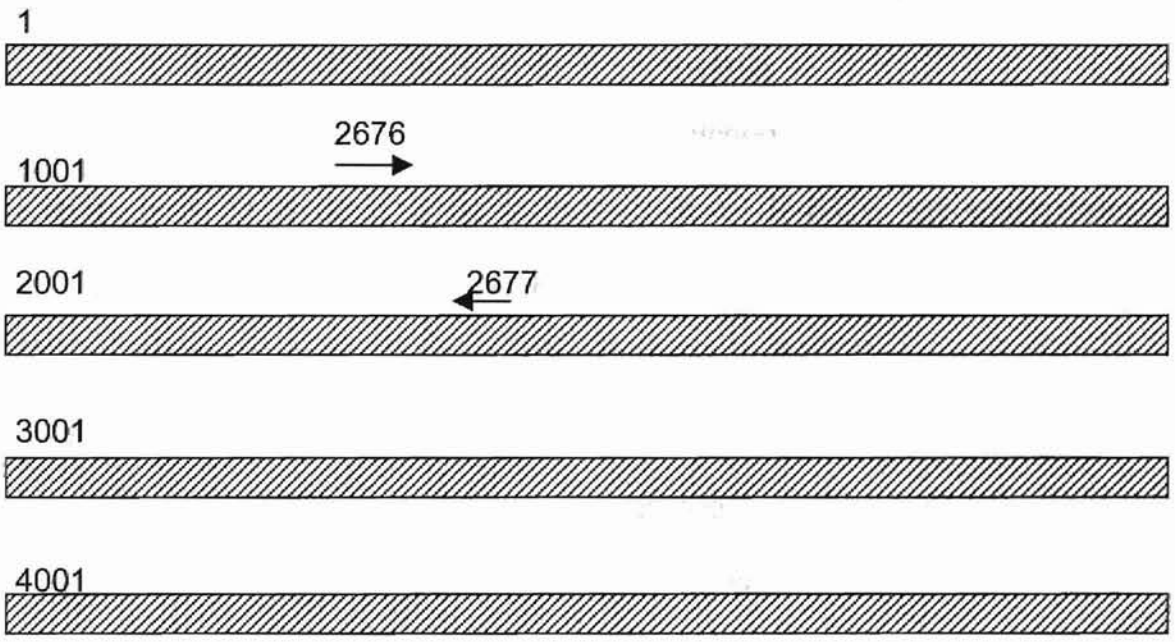

Fig. 5: Representation of the BM-3 gene from *B. megaterium*. 2676 is a specific upstream primer and 2677 is a specific downstream primer.

Possibly, the predicted size fragment, using primers 2533 and 3576, for *E. coli* was not generated due to competing nucleic material. Therefore, optimization of the PCR procedure was attempted by isolating only mRNA from

the bacteria. The RT-PCR technique was used to isolate mRNA. The rationale behind this approach is some bacterial species produce a poly A tail, including *E. coli*. Therefore, it is possible to only collect mRNA containing a poly A tail, while eliminating other nucleic acid material that may interfere in the PCR reaction. The mRNA approach resulted in the formation of three bands, 200, 350, and 800 bp using the 3576 and 2533 primers. The PCR sample containing all three bands were ligated into the TA cloning vector (Invitrogen). The plasmids were then transformed into competent cells. Ten positive colonies were isolated. The plasmid was extracted and cut with an *Eco*RI restriction enzyme, in order to release the insert. Three samples contained inserts and were 800 bp in length. The TA vector containing the 800 bp fragment was purified and sequenced. A BLASTN was executed and the sequence matched to the *E. coli* chromosome with a score of 158 with 313 bits and an e-value of 2e-85, which is 99% identical to section 31 of 400 from *E. coli*. Performing a BLASTX resulted in a score of 387 and 167 bits and an e-value of 1e-42, which is 98% identical to the beta-D-galactosidase protein of *E. coli*. To determine if the protein beta-D-galactosidase from *E. coli* matched section 31 of 400 on the *E. coli* chromosome, a partial amino acid sequence from beta-D-galactosidase was compared. The TBLASTN results were identical to section 31 of 400 for *E. coli*.

The 800 bp product information from genomic DNA and mRNA were different. The primer bound to different regions on the *E. coli* chromosomal DNA and cDNA. It was concluded that the primers did not have a high homology to the regions they bound, thereby, non-specific binding occurred. To determine if

E. coli contained sequences similar to cytochrome P450, the P450CAM sequence was compared to the *E. coli* chromosome database (recent sequence of the entire genome). A BLASTN search did not result in any significant homology between P450CAM and the *E. coli* chromosome. In addition, the BLASTX results did not result in any significant homology to *E. coli* proteins.

Discussion

Different methods were successfully developed in order to screen intestinal bacteria for the presence of cytochrome P450. These methods involved designing specific conserved primers, and developing procedures for the PCR portion of the study. The initial investigation tested twelve different ATCC intestinal bacterial strains. The ATCC strains were selected based on their importance as major human intestinal flora ([25]). The results from the initial investigation determined that *E. coli* was the only strain that produced a predicted size PCR product. Therefore, *E.* coli was used to develop and test the different methods, as well as, determining whether the cytochrome P450 gene was indeed present in the bacterium. The development of these methods included testing whole cells, chromosomal, and cDNA amplification. In addition, different methods of PCR product isolation and analysis were developed.

There were three methods tested that involved preparing DNA for the PCR reaction. All three methods were tested using the same PCR reaction conditions. It was concluded that the whole cell PCR method did not yield a clear PCR fragment. Thus, sequence information could not be obtained. The chromosomal extraction method resulted in an increase in PCR product generation. In an attempt to improve the PCR reaction, a cleaner DNA preparation was attempted. Messenger RNA was initially isolated and cDNA was synthesized ([26]). The cDNA then served as the template, thereby, eliminating the presence of other nucleic acid material that would interfere with the PCR

reaction. The cDNA method did result in the amplification of unique DNA amplified fragments that were not present in the chromosomal preparation.

Once the PCR products were produced, two methods of PCR fragment preparation for purpose of sequencing were tested. The evaluation criteria for the two methods were based on the generation of clear sequence information. The two methods tested were 1) the electro-elution method, and 2) the TA cloning method. The electro-elution method was more accurate when a single band was clearly isolated from other bands in a lane. In comparison, the TA cloning kit produced a higher yield of PCR product, thereby, making it more efficient for PCR sequencing.

The primers used in the study were homologous to P450 conserved regions. Unfortunately, the primers did not produce the predicted PCR fragment from the selected strain, nor did the PCR fragment contain sequences similar to existing P450 families. Therefore, either no P450 was present in *E. coli* or a new P450 family may be present.

A comparison of the entire *P. putida* P450CAM nucleotide sequence with the entire *E. coli* genome revealed no significant homology between the two. A comparison of the amino acid sequence from P450CAM with *E. coli* proteins also did not show any significant homology. The results support a review by Munro ([27]), which reported that *E. coli* appeared to be devoid of P450. However, an *E. coli* electron-transfer system, capable of supporting the function of a heterologously-expressed P450, does exist.

Recent completion of the entire genome sequence for the *E. coli* K-12 strain revealed that no cytochrome P450 gene from an existing family was present ([28]). It should be noted that the *E. coli* strain used in this study was isolated from human intestinal feces ([25]) and is different from the *E. coli* K-12 strain. To determine if the intestinal strain used in this study contains a new family of P450, the entire genome must be sequenced or the protein isolated and activity demonstrated.

Chapter III

Phenobarbital Induction in Intestinal Microflora

Introduction

Cytochrome P450 is inducible by a number of different compounds (e.g. xenobiotics, nitrates, and azo-dyes). The induction of cytochrome P450 in turn can result in the metabolism of a number of different man-made or foreign compounds called xenobiotics (drugs, dyes, pesticides, etc.). Therefore, one method of identifying new cytochrome P450s is to induce the protein with existing inducers such as phenobarbital. Since phenobarbital is a common inducer for mammalian and bacterial systems, it was used in this study as an inducer to screen a number of intestinal bacteria for the presence of cytochrome P450.

Since the enzyme systems in intestinal bacteria (over 400 species) have not been fully characterized, it is possible that cytochrome P450 may exist in intestinal bacteria. In addition, there has not been an extensive study to determine the presence of cytochrome P450 in major intestinal strains such as *Bacteroides spp.*, *Bifidobacterium spp.*, *Fusobacterium spp.* and *Clostridium spp.*.

To determine the presence of cytochrome P450 in major strains of intestinal flora, eighteen bacterial strains were grown in the presence of phenobarbital. The strains were selected based on reports by Moore regarding their importance to normal human physiology ([25]).

Methods and Materials

Preparation of anaerobic media: Two different types of media were used 1.) (Tryptone, Yeast Extract and Glucose (TYG) and 2.) Brain Heart Infusion (BHI)). TYG: For 500 ml of medium, the following were added- 5 g of tryptone, 2.5 g yeast extract, 425 ml dH_2O, 12.5 ml salt solution 1 (3 g K_2HPO_4 in 1 liter water), 12.5 ml salt solution 2 (3.54 g KH_2PO_4, 9 g $(NH_4)_2SO_4$, 0.9 g NaCl, 1.9 g $MgSO_4$ in 500 ml water), 12.5 ml $CaCl_2$ solution (0.6 g $CaCl_2$ in 1 liter water), 2.0 ml hemin (50 mg hemin, 1 ml 1N NaOH in 100 ml water), 1 ml Wolfe's mineral solution(2X) (1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \bullet 7H_2O$, 0.5 g $MnSO_4 \bullet H_2O$, 1 g NaCl, 0.1 g $FeSO_4 \bullet 7H_2O$, 0.1 g $CoCl_2 \bullet 6H_2O$, 0.1 g $ZnSO_4 \bullet 7H_2O$, 0.01 g $CuSO_4 \bullet 5H_2O$, 0.01g $AlK(SO_4)_2 \bullet 12H_2O$, 0.01 g H_3BO_3, 0.01g $Na_2MoO_4 \bullet 2H_2O$ into 500 ml water) and 5.0 ml resazurin (0.25 g resazurin in 500 ml water) were added. The medium was then boiled using a microwave for 5 minutes and degassed (bubbled) with N_2 for 30 minutes. When the TYG was approximately 40°C, 5 ml vitamin K solution (50 mg menadione in 100 ml 95% EtOH) and 0.3 g cysteine-HCl were added. The pH was adjusted to 7.0. The solution was then bubbled for another 10 minutes. In a separate bottle, 10 ml H_2O, 10 ml volatile fatty acids (42.5 ml acetic acid, 15.0 ml propionic acid, 10.0 ml butyric acid, 2.5 ml isobutyric acid, 2.5 ml valeric acid, 2.5 ml isovaleric acid and 2.5 ml methylbutryic acid with the pH adjusted to 9.5 and the volume made up to 500 ml with ddH_2O) and 0.2 g sodium carbonate were added to a 100 ml bottle. The bottle was sealed and autoclaved with the TYG solution.

After autoclaving, N_2 was bubbled into the TYG solution until the solution was less than 50°C. Aseptically, 20 ml 20% anaerobic glucose, 20 ml volatile fatty acid, 10 ml reducing agent (110 ml distilled water microwaved for two minutes, gassed with N_2 until room temperature, then 2.5 g cysteine-HCl was added, the pH adjusted to 9.0 using 5 N NaOH, and 2.5 g $Na_2S \bullet 9H_2O$ were added and placed in N_2 equilibrated bottles, and autoclaved) and 1 ml 10X vitamins (5 mg riboflavin, 100 μg Vitamin B_{12}, 5 mg pyridoxine(B_6), 5 mg pyridoxal, 5 mg pyridoxamine, 5 mg pantothenate-Ca, 3 mg nicotinic acid, 3 mg nicotinamide, 2 mg biotin, 2 mg folic acid, 5 mg thiamine(B_1), 5 mg thioctic acid and 5 mg p-amino benzoic acid were added to 50 ml distilled water and filter sterilized (0.2μm) and placed into N_2 equilibrated bottles and sealed) were added to the solution, completing the TYG medium. The medium was placed in an anaerobic chamber and incubated at 37°C overnight to test for contamination.

BHI medium preparation was similar to TYG, except tryptone was replaced with 18 g brain heart infusion and 12.5 g yeast extract was added instead of 2.5 g from the TYG recipe.

Inoculation of media: From an active sample of bacteria growing (24 –48 hrs) in Chopped Meat Carbohydrate (Anaerobe Systems Morgan Hill, CA), 100 μl was transferred to 5 ml of BHI and incubated overnight at 37°C. Phenobarbital (inducer) was filter sterilized (0.2μm) and added for a final concentration of 5 mM into one of two bottles of anaerobic media (according to Table 4). The overnight culture was divided into two equal parts and inoculated into both bottles (with and

without inducer). The sample was incubated for 24 to 48 hours at 37°C or until visually turbid.

Protein Extraction: The 500 ml culture of bacteria (induced or non-induced) was separated into two equal parts in N_2 equilibrated 250 ml Nalgene anaerobic bottles. Using a Sorvall centrifuge (GSA-13 rotor, Newtown, CT), the samples were centrifuged at 10,000 rpm for 15 minutes at 4°C. After centrifugation, the bottles were placed back into the anaerobic chamber and the supernatant removed. Each pellet was washed with 10 ml of anaerobic Mops buffer (100 mM Mops (pH 7.3), 10% glycerol, 0.2 mM dithiothreitol, and 1mM EDTA) and combined for a total of approximately 20 ml Mops buffer. The suspension was then transferred to a N_2-equilibrated 50 ml centrifuge tube and sealed with an anaerobic cap. The suspension was again centrifuged at 10,000 rpm for 15 minutes at 4°C. In the anaerobic chamber, the supernatant was removed and 10 ml of fresh Mops buffer was added and the pellet was re-suspended. For Gram-positive bacteria, 10µl mutanolysin was added and the sample incubated for two hours at 37°C. The suspension was placed on ice for 2 minutes. Once cooled, the sample (Gram-positive or Gram-negative) was sonicated under N_2 gas. Using a flat tip sonicator probe (Branson Sonic Power Co., Danburry, CT), the sample was pulsed for 2-seconds, 25 times. The sample was placed on ice for 2 minutes and the above step was repeated twice. In the anaerobic chamber, the sample was transferred to an ultra-centrifuge tube that was equilibrated with N_2. The sample was centrifuged in the ultra-centrifuge at 31,000 rpm for 35 minutes at 4°C in a Ti-70 rotor. In the anaerobic chamber, the

supernatant was fractionated into 1.5 ml anaerobic tubes that were equilibrated with N_2. The supernatants were labeled fraction 1 and stored at -80°C. A 10 ml amount of Mops buffer containing CHAPS (final concentration of 0.5%) was added to the pellet. The sample was capped and stirred overnight at 4°C with a metal stir bar and magnetic stirrer. The ultra-centrifugation and fractionation steps were repeated. The second fraction was labeled fraction 2 and also stored at -80°C.

Protein determination: To determine the protein concentration, two protein assays were used: 1) Bio-Rad *DC* (Detergent Compatible) (Bio-Rad, Hercules CA) and 2) the Folin reaction. (Note: two different assays were used, due to available chemicals). The Folin reaction was comprised of four reagents. Reagent A (2 g of Na_2CO_3 in 100 ml of 0.1 N NaOH) was added to reagent B (0.05 g of $CuSO_4 \bullet 5\ H_2O$ in 10 ml of a 1% (w/v) solution of sodium tartrate) at a 50:1 ratio to make reagent C. Reagent D was 1 N Folin-Phenol. At total of 100µl of protein (fraction 1 and fraction 2) was added to 300µl of water. Then, 2 ml of reagent C was added and vortexed. This mixture was incubated at room temperature for 10 minutes. Then 200 µl of reagent D was added, vortexed, and incubated for 30 minutes. The absorbance was analyzed at 750 nm using a Shimadzu spectrophotometer (Columbia, MD). The Bio-Rad DC assay followed the manufactures protocol.

CO difference spectrum: The CO difference spectrum assay was used to detect a 450nm peak from the protein fractions ([29]). To determine the CO difference absorbance spectra, 1 ml of the protein fraction (1 and 2) were

thawed. Sodium hydrosulfite (final concentration of 10 mM) was added to the fraction. The fraction was inverted 5 times and the absorbance was scanned from 400 to 600 nm in order to establish a base line. CO was then bubbled through the sample for approximately 30 seconds. The absorbance of the sample was then scanned again. The sample was rescanned 5 minutes later.

Protein preparation for SDS-PAGE: Based on the protein determination assay, four volumes (600 μl) methanol, 1 volume (150μl) chloroform and 3 volumes (450 μl) distilled water were added to 100 μg of the protein sample. The sample was vortexed for 30 seconds and centrifuged for 5 minutes at 14,000 rpm (Eppendorf model 5415c). The upper layer was discarded, leaving the interface and lower layer. To the interface and lower layer, 300 μl methanol was added and inverted 5 times. Again, the sample was centrifuged for 5 minutes at 14,000 rpm. The supernatant was removed and the subsequent pellet dried (Speed-Vac) for 10 minutes to remove residual methanol. To the dry pellet, 20 μl distilled water and 5μl 4X tracking dye (3.8 ml distilled water, 1 ml 0.5 M tris-HCl at a pH 6.8, 800 μl glycerol, 1.6 ml 10% (w/v) SDS, 400 μl β-mercaptoethanol and 0.05% (w/v) bromophenol blue) were added. The sample was boiled for 5 minutes, vortexed, and then centrifuged for 2 minutes at 14,000 rpm to pellet insoluble debris. All 25 μl of the supernatant was loaded onto an SDS-PAGE gel.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): For the 10% running gel, 4 ml of lower buffer (1.5 M Tris-HCl at a pH 8.8) was added to 4 ml 40% acrylamide:bis (Bio-Rad at a ratio of 39:1 and 37.5:1), 7.67 ml nanopure water, 160 μl 10%SDS, 160 μl 10% APS (ammonium

persulfate) and 16 µl TEMED (Bio-Rad). For the 5 % stacking gel, 750 µl upper buffer (0.5 M Tris-HCl at a pH 6.8) was added to 750 µl 40% acrylamide, 4.38 ml nanopure water, 60 µl 10% SDS, 60 µl 10% APS, and 6 µl N,N,N,N`-Tetra-methyl-ethylenediamine (TEMED). The separating gel was first poured and overlaid with a 50% water-saturated butanol. After the gel polymerized, the butanol was rinsed off with distilled water and the stacking gel poured on top. A running buffer was made at a concentration of 5X (60 g Tris base, 288 g glycine and 20 g SDS in 4 L water) and diluted to a 1X working solution. The electrophoresis was carried out for approximately 45 minutes at 80 volts. After electrophoresis, the gel was stained with coomassie stain (40 ml methanol, 10 ml acetic acid, 0.1 g coomassie blue R-250 and 50 ml distilled water) for at least two hours and destained (50% methanol and 50% distilled water) until the background was removed. The gel was then dried on an air dryer (Bio-Rad).

Species Name	ATCC #	Location*	Gram positive/ negative	Media used
Bacteroides fragilis	25285	Colon	-	TYG
Bacteroides stercoris	43183	Colon	-	TYG
Bifidobacterium adolescentis	15703	Colon	+	TYG
Bifidobacterium bifidum	15696	Colon	+	TYG
Bifidobacterium breve	15700	Colon	+	TYG
Bifidobacterium infantis	15697	Colon	+	TYG
Bifidobacterium longum	15707	Colon	+	TYG
Clostridium perfringens	19574	Colon	+	TYG
Enterococcus faecalis	27274	Colon	+	TYG
Enterococcus faecium	6569	Colon	+	TYG
Escherichia coli	11698	Colon	-	TYG
Eubacterium eligans	27750	Colon	+	BHI
Eubacterium rectale	33656	Colon	+	BHI
Lactobacillus acidophilus	4356	Stomach	+	TYG
Ruminococcus productus	27340	Colon	+	BHI
Staphylococcus aureus	13150	Stomach	+	TYG
Streptococcus intestinalis	43492	Colon	+	BHI

Table 4. Bacterial strain grown and tested for the presence of cytochrome P450 induced with phenobarbital. All strains with the exception of *S. intestinalis* were isolated from the human intestine. *S. intestinalis* was isolated from swine. The strains were obtained from ATCC and selected based on reports by Moore ([25], * [17], [30]).

Results

Each strain was grown anaerobically to saturation (stationary phase) in the presence and absence of 5mM phenobarbital. The average time of growth was 24 hours. Based on visual turbidity analysis of the plus and minus phenobarbital samples, phenobarbital did not appear to inhibit the growth of the bacterial cultures exposed to phenobarbital. Two bacterial fractions, fraction 1 from the cytoplasm and fraction 2 from a membrane-bound soluble preparation were collected under anaerobic conditions. The protein fractions were isolated under anaerobic conditions in order to reduce the level of free oxygen molecules interaction with the anaerobic proteins. Many anaerobic bacteria lose their reductive activity after oxygen exposure. Since activity of the P450 enzyme and the 450 nm peak are coupled, the level of oxygen exposure to the samples was limited. The reduced carbon monoxide (CO) difference spectrum was performed on all fractions. Table 5 summarizes the CO difference data for all strains tested.

The data for the induced fraction 1 samples were the following. *Bifidobacterium infantis* produced a peak at 584 nm, *Clostridium perfringens* produced two peaks at 424 and 581 nm, *Ruminococcus productus* produced a peak at 575 nm, and *Streptococcus intestinalis* produced two peaks at 416 and 572 nm. The data for the non-induced fraction 1 samples were the following. *Bacteroides stercoris* produced two peaks at 415 and 575 nm and *Bifidobacterium longum* produced a peak at 480 nm.

The data for the induced fraction 2 sample were the following. *Clostridium perfringens* produced a peak at 420 nm, but not a peak at 581 nm as seen with the induced fraction 1. *S. intestinalis* produced two peaks at 416 and 575 nm, which was similar to the induced fraction 1 sample. *R. productus* did not produce a peak in induced fraction 2, compared to a 575 peak in the induced fraction 1 sample. There were no peaks observed in fraction 2 non-induced samples.

Species Name	Protein Fraction 1		Protein Fraction 2	
	Non-Induced (nm)	Induced (nm)	Non-Induced (nm)	Induced (nm)
Bacteroides fragilis	None	None	None	None
Bacteroides stercoris	415, 575	None	None	None
Bifidobacterium adolescentis	None	None	None	None
Bifidobacterium bifidum	None	None	None	None
Bifidobacterium breve	None	None	None	None
Bifidobacterium infantis	None	584	None	None
Bifidobacterium longum	480, 573	573	None	None
Clostridium perfringens	None	424, 581	None	420
Enterococcus faecalis	None	None	None	None
Enterococcus faecium	None	None	None	None
Escherichia coli	None	None	None	None
Eubacterium eligans	None	None	None	None
Eubacterium rectale	None	None	None	None
Lactobacillus acidophilus	None	None	None	None
Ruminococcus productus	None	575	None	None
Staphylococcus aureus	None	None	None	None
Streptococcus intestinalis	None	416, 572	None	416, 575

Table 5. Protein fractions. The numbers represent wavelengths (nm) for non-induced and induced proteins based on the reduced CO difference spectrum assay. Fraction 1 represents proteins from the cytoplasm and fraction 2 represents proteins from the membrane.

Species Name	Repression molecular weight (kDa)	Induction molecular weight (kDa)
Bacteroides fragilis	None	None
Bacteroides stercoris	47	None
Bifidobacterium adolescentis	None	38 and 42
Bifidobacterium bifidum	66	None
Bifidobacterium breve	None	28
Bifidobacterium infantis	25	20
Bifidobacterium longum	28	35
Clostridium perfringens	None	None
Enterococcus faecalis	None	None
Enterococcus faecium	None	None
Escherichia coli	None	None
Eubacterium eligans	30	66
Eubacterium rectale	None	None
Lactobacillus acidophilus	None	None
Ruminococcus productus	None	None
Staphylococcus aureus	None	None
Streptococcus intestinalis	None	68

Table 6. The SDS-PAGE results of the protein profile. Bacterial strains that had an induction or a repression of proteins with 5mM phenobarbital.

Both non-induced and induced samples from both the cytoplasm and membrane-bound preparations were analyzed by one-dimensional SDS-PAGE. Table 6 summarizes the results for all bacterial strains tested. The induced proteins produced the following results. *B. longum* produced a protein band at a molecular weight of 35 kDa for fraction 1 (Table 6 and Fig. 6A lane 2), *B. infantis* produced a protein band at 20 kDa for fraction 1 (Table 6 and Fig. 6B lane 3), *B. breve* produced a protein band at 28 kDa for fraction 2 (Table 6 and Fig. 6B lane 9), *B. adolescentis* produced two protein bands at 38- and 42 kDa for fraction 2 (Table 6 and Fig. 7A lane 4), *E. eligans* produced a protein band at 66 kDa for fraction 2 (Table 6 and Fig.7B lane 4), and *S. intestinalis* produced a protein band at 68 kDa for fraction 2 (Table 6 and Fig.7C lane 4). The arrows for each figure show the protein described. The repressed proteins are listed in Table 6.

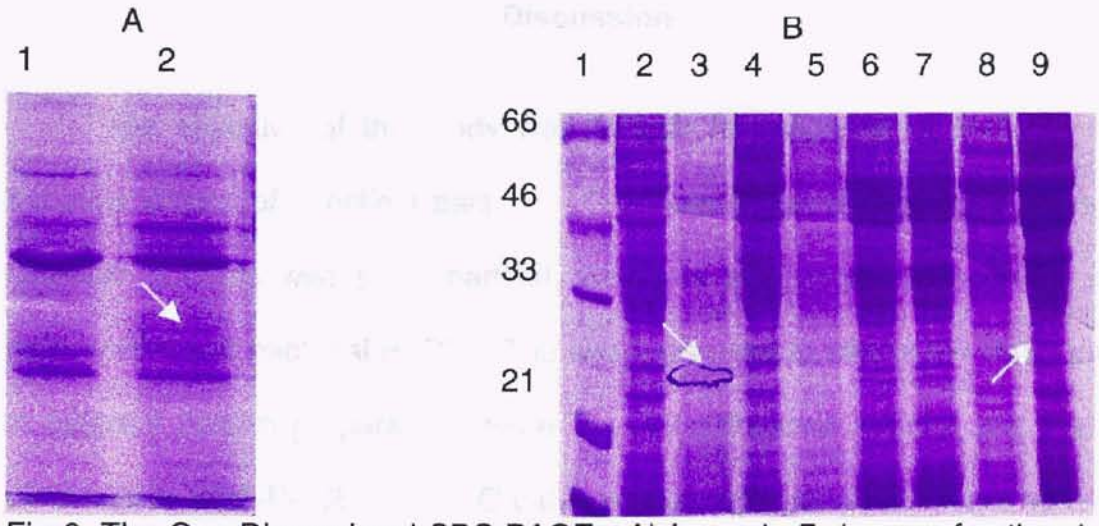

Fig 6. The One-Dimensional SDS-PAGE. A) Lane 1, *B. longum* fraction 1 non-induced; and lane 2, induced fraction 1. Lane 2 generated an induced protein near 35 kDa. B) Lane 1 molecular weight marker. Lane 2, *B. infantis* fraction 1 non-induced; lane 3, fraction 1 induced; lane 4, fraction 2 non-induced; and lane 5 fraction 2 induced. Lane 3 generated an induced protein near 20 kDa. Lane 6, *B. breve* fraction 1 non-induced; lane 7 fraction 1 induced; lane 8 fraction 2 non-induced; and lane 9 fraction 2 induced. Lane 9 generated an induced protein near 28 kDa. The repressed proteins are not shown.

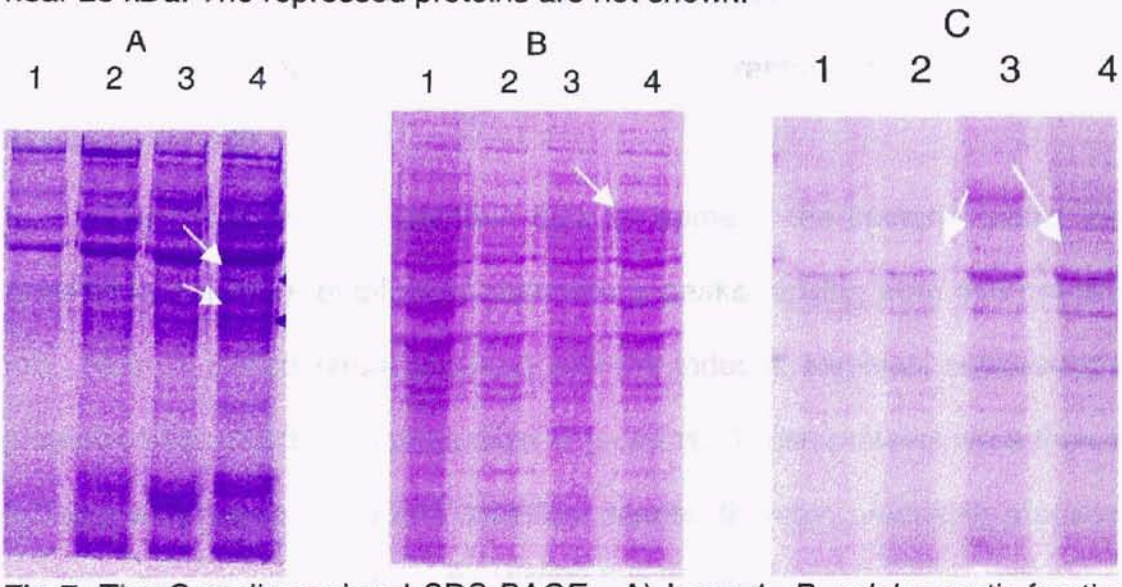

Fig 7. The One-dimensional SDS-PAGE. A) Lane 1, *B. adolescentis* fraction 1 non-induced; lane 2 fraction 1 induced; lane 3 fraction 2 non-induced; and 4 fraction 2 induced. Lane 4 generated two induced protein bands near 42- and 38 kDa. B) Lane 1, *E. eligans* fraction 1 non-induced; lane 2 fraction 1 induced; lane 3 fraction 2 non-induced; and 4 fraction 2 induced. Lane 4 generated an inducible band near 66 kDa. C) Lane 1, *S. intestinalis* fraction 1 non-induced; lane 2 fraction 1 induced; lane 3 fraction 2 non-induced; and 4 fraction 2 induced. Lane 4 generated an induced protein near 68 kDa. The molecular weight marker is not shown. The repressed proteins are not shown.

Discussion

The objective of the study was to look for evidence of P450 in major bacterial strains of intestinal bacteria. This was accomplished by inducing the bacterial cultures with phenobarbital, which is a common inducer for many mammalian and bacterial P450s. The two tests used to detect the P450 protein in different protein preparations were the CO difference spectra and the one-dimensional SDS-PAGE. The CO difference absorbance spectra test was used to determine the absorbance peak at or near the predicted 450 nm region or near the 420 nm region. The one-dimensional SDS-PAGE was used to analyze the protein profile of induced and non-induced samples from the different fraction preparations. The SDS-PAGE was also used to determine the presence of an induced protein near the 45-55kDa or 119kDa range, which is indicative of cytochrome P450.

The CO difference spectrum data for some of the bacterial stains tested resulted in a number of different absorbance peaks ranging from 415 nm to 584 nm. Most of the spectrum occurred with the induced samples, suggesting that phenobarbital affected the production of proteins. These proteins were then able to react with the reducing and oxidation agents, thereby, producing a spectrum profile. The spectrums from non-induced samples were probably caused by constitutive proteins, as phenobarbital was not present.

The presence of phenobarbital in the cultures affected the expression of some of the bacterial stains tested, as the proteins expressed ranged in size from 20kDa to 68 kDa. Interestingly, phenobarbital caused the repression of some

proteins in some of the bacterial strains tested. The molecular mechanism associated with the observed responses is complex, as it is not known if phenobarbital acted as a positive regulator or as a negative regulator. What is known is that phenobarbital influenced the expression of a number of proteins in different bacterial cultures. Future studies to identify these induced proteins would shed some light as what proteins are affected by phenobarbital.

C. perfringens and *S. intestinalis* produced a CO difference spectrum near 420 nm, which is similar to denatured cytochrome P450. However, only *S. intestinalis* produced an induced protein near 55 kDa. Therefore *S. intestinalis* was selected for further characterization.

Chapter IV

The Identification of Two Proteins From *Streptococcus intestinalis*

Introduction

Streptococcus intestinalis is a gram-positive anaerobe. It is a naturally-occurring ureolytic streptococcus isolated from swine feces. It was first isolated in 1988 by Robinson *et. al.* ([31]) while studying ammonia production by bacteria in urea. In 1999, however, Vandamme *et. al.*([32]) determined that *Streptococcus intestinalis* and *Streptococcus alactolyticus* were phenotypically indistinguishable, based on the whole-cell protein electrophoresis and biochemical tests.

S. intestinalis is an intestinal microflora. As with other intestinal strains, its metabolic capacity, is not fully understood. This study determined the ability of *S. intestinalis* to grow in the presence of 5mM phenobarbital, a compound used to induce cytochrome P450. The hypothesis is that intestinal bacteria, like *S. intestinalis*, possess a cytochrome P450 system that is involved in metabolizing different xenobiotics thereby influencing the expression of hepatic cytochrome P450. It has been shown in animal studies that intestinal flora can influence the expression of hepatic cytochrome ([33]). But the presence of cytochrome P450 or cytochrome P450-like proteins in intestinal bacteria has not been extensively studied.

In the presence of phenobarbital, the growth rate of *S. intestinalis* was analyzed. In addition, the presence of induced proteins was determined based on SDS-PAGE analysis. Induced proteins from *S. intestinalis* were purified by 2-

D SDS-PAGE and N-terminally sequenced. The induced proteins resembled OprF , a porin protein, and GroEL, a heat shock protein, from *Pseudomonas spp.*

Methods and Materials

Bacterial strain: *Streptococcus intestinalis* (ATCC # 43492) was purchased from The American Type Culture Collection.

Culture media: BHI: For 500 ml of medium, 18 g of Brain Heart Infusion, 2.5 g yeast extract, 425 ml dH_2O, 12.5 ml salt solution 1 (3 g K_2HPO_4 in 1 liter water), 12.5 ml salt solution 2 (3.54 g KH_2PO_4, 9 g $(NH_4)_2SO_4$, 0.9 g NaCl, 1.9 g $MgSO_4$ in 500 ml water), 12.5 ml $CaCl_2$ solution (0.6 g $CaCl_2$ in 1 liter water), 2.0 ml hemin (50 mg hemin, 1 ml 1N NaOH in 100 ml water), 1 ml Wolfe's mineral solution(2X) (1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \bullet 7H_2O$, 0.5 g $MnSO_4 \bullet H_2O$, 1 g NaCl, 0.1 g $FeSO_4 \bullet 7H_2O$, 0.1 g $CoCl_2 \bullet 6H_2O$, 0.1 g $ZnSO_4 \bullet 7H_2O$, 0.01 g $CuSO_4 \bullet 5H_2O$, 0.01g $AlK(SO_4)_2 \bullet 12H_2O$, 0.01 g H_3BO_3, 0.01g $Na_2MoO_4 \bullet 2H_2O$ into 500 ml water) and 5.0 ml resazurin (0.25 g resazurin in 500 ml water) were added. The medium was then boiled using a microwave for 5 minutes and degassed (bubbled) with N_2 for 30 minutes. When the BHI was approximately 40°C, 5 ml vitamin K solution (50 mg menadione in 100 ml 95% EtOH) and 0.3 g cysteine-HCl were added. The pH was adjusted to 7.0. The solution was then bubbled for another 10 minutes. A total of 10 ml H_2O with 10 ml volatile fatty acids (42.5 ml acetic acid, 15.0 ml propionic acid, 10.0 ml butyric acid, 2.5 ml isobutyric acid, 2.5 ml valeric acid, 2.5 ml isovaleric acid and 2.5 ml methylbutryic acid with the pH adjusted to 9.5 and the volume made up to 500 ml with ddH_2O) and 0.2 g sodium carbonate were added into a 100 ml bottle. The bottle was sealed and autoclaved with the BHI solution.

After autoclaving, the N_2 was bubbled into the BHI solution until the solution was less than 50°C. Aseptically, 20 ml 20% anaerobic glucose, 20 ml volatile fatty acid, 10 ml reducing agent (110 ml distilled water microwaved for two minutes, gassed with N_2 until room temperature, then 2.5 g cysteine-HCl added, the pH adjusted to 9.0 using 5 N NaOH, and 2.5 g $Na_2S \bullet 9H_2O$ were added and placed in N_2 equilibrated bottles, and autoclaved) and 1 ml 10X vitamins (5 mg riboflavin, 100 μg Vitamin B_{12}, 5 mg pyridoxine(B_6), 5 mg pyridoxal, 5 mg pyridoxamine, 5 mg pantothenate-Ca, 3 mg nicotinic acid, 3 mg nicotinamide, 2 mg biotin, 2 mg folic acid, 5 mg thiamine(B_1), 5 mg thioctic acid and 5 mg p-amino benzoic acid were added to 50 ml distilled water and filter sterilized (0.2μm) and placed into N_2 equilibrated bottles and sealed) were added to the solution, competing the BHI medium. The medium was placed in an anaerobic chamber and incubated at 37°C overnight to test for contamination.

Growth curve: Two 5 ml tubes of BHI were inoculated with 100μl of an overnight culture of *S. intestinalis*. Phenobarbital was added (final concentration 5 mM) to one tube. A spectronic 20D (Milton Roy, Rochester, NY) spectrophotometer was used to measure the optical density at 590 nm. A separate tube containing only media served as a blank control. Measurements were recorded for both actively-growing cultures every 30 minutes for up to six hours.

Protein Extraction: The 500 ml culture, induced or non-induced, of bacteria was separated into two equal parts in N_2 equilibrated 250 ml Nalgene anaerobic bottles. Using a Sorvall centrifuge (GSA-13 rotor, Newtown, CT), the samples

were centrifuged at 10,000 rpm for 15 minutes at 4°C. After centrifugation, the bottles were placed back into the anaerobic chamber and the supernatant was removed. Each pellet was washed with 10 ml of the anaerobic Mops buffer (100 mM Mops (pH 7.3), 10% glycerol, 0.2 mM dithiothreitol, and 1mM EDTA) and combined for a total of 20 ml Mops buffer. The suspension was then transferred to a N_2-equilibrated 50 ml centrifuge tube and sealed with an anaerobic cap. The suspension was again centrifuged at 10,000 rpm for 15 minutes at 4°C. In the anaerobic chamber the supernatant was removed and 10 ml of fresh Mops buffer was added and the pellet was re-suspended and incubated for two hours at 37°C with 10µl mutanolysin. The suspension was placed on ice for 2 minutes. Once cooled, the sample was sonicated under N_2 gas. Using a flat tip sonicator probe, the pulse was set for 2 seconds for a total of 25 times. The sample was placed on ice for 2 minutes and the above step was repeated twice more. In the anaerobic chamber, the sample was transferred to an ultra-centrifuge tube that was equilibrated with N_2. The sample was centrifuged in the ultra-centrifuge, at 31,000 rpm for 35 minutes at 4°C in a Ti-70 rotor. In the anaerobic chamber the supernatant was fractionated into 1.5 ml anaerobic tubes that were equilibrated with N_2. The supernatants were labeled fraction 1 and stored at -80°C. CHAPS (0.05 g) and 10 ml MOPS buffer were added to the pellet. The sample was stirred overnight at 4°C with a metal stir bar and magnetic stirrer. The ultra-centrifugation and fractionation was repeated.

Protein determination: To determine the protein concentration, the Bio-Rad *DC* (Detergent Compatible) protein assay from Bio-Rad was used. The

absorbance was analyzed at 750 nm using a Shimadzu spectrophotometer (Columbia, MD).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): For the 10% running gel, 4 ml of lower buffer (1.5 M Tris-HCl at a pH 8.8) was added to 4 ml 40% acrylamide (Bio-Rad at a ratio of 39:1 and 37.5:1), 7.67 ml nanopure water, 160 µl 10%SDS, 160 µl 10% APS (ammonium persulfate) and 16 µl TEMED (Bio-Rad). For the 5 % stacking gel, 750 µl upper buffer (0.5 M Tris-HCl at a pH 6.8) was added to 750 µl 40% acrylamide, 4.38 ml nanopure water, 60 µl 10% SDS, 60 µl 10% APS, and 6 µl TEMED. The separating gel was first poured and overlaid with 50% water-saturated butanol. After the gel polymerized, the butanol was rinsed off with distilled water and the stacking gel poured on top. A running buffer was made at a concentration of 5X (60 g tris base, 288 g glycine and 20 g SDS in 4 L water) and diluted to 1X as a working solution. The electrophoresis was carried out for approximately 45 minutes at 80 volts. After electrophoresis, the gel was stained with a coomassie stain (40 ml methanol, 10 ml acetic acid, 0.1 g coomassie blue R-250 and 50 ml distilled water). The gel was stained for at least two hours and destained (50% methanol and 50% distilled water) until the background was removed. The gel was then dried on an air dryer (Bio-Rad).

2-D gel electrophoresis: For the first dimension gel, 5.5 g of urea was added to 1.33 ml 40% acrylamide (Bio-Rad at a ratio of 37.5:1) along with 2.0 ml 10% Triton X-100 and 0.400 ml Bio-Lyte 3/10 ampholyte and 0.100 ml Bio-Lyte 5/7 ampholyte and 1.97 ml distilled water. The solution was warmed, vortexed

and degassed for 15 minutes. For polymerization, 10 μl of 10% ammonium persulfate and 10 μl TEMED were added and poured in the casting tube until it was three-fourths of the length of the capillary tubes. (Note: one end of the casting tube was sealed with several layers of parafilm and the capillary gel tubes were then placed, with the blue end up, in the casting tube). After polymerization, the parafilm was slowly removed from the bottom of the casting tube and the capillary tubes removed. The tubes were connected to the apparatus and the bottoms of the tubes were rinsed with lower buffer (0.068 % (v/v) H_3PO_4). Each sample reservoir was filled with degassed upper buffer (2 M NaOH). A stir bar was placed in the lower chamber along with the tube gel adapter. The lower buffer was added to the lower chamber, up to the blue line on the glass gel tubes. The upper chamber was filled with upper buffer, just above the sample reservoirs. The gel tubes were then electrophoresed at 200 V for 10 minutes, 300 V for 15 minutes and 400 V for 15 minutes as a pre-electrophoresis step. After the pre-electrophoresis was completed, the upper and lower buffers were discarded and replaced with fresh upper and lower buffer. A 10 μl amount of the sample was added to an equal volume of the first dimension sample buffer (8M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte and 0.4% Bio-Lyte 3/10 ampholyte) and incubated at room temperature for 15 minutes and then loaded into the tube gel. The sample was then overlaid with a 40 μl overlay buffer (8.5 M urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte and bromophenol blue). The sample was then electrophoresed for 10 minutes at 500 V and 750 V for 3.5

hours. The gel was removed from the tube with a 1.0 ml syringe attached to the white end of a tube gel ejector. The capillary tube was inserted into the colored end of the unit. The tube gel was extruded onto a piece of parafilm and equilibrated with SDS sample buffer (0.0625 M Tris HCl, pH 6.8, 2.3% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol, 10% glycerol (w/v), bromophenol blue and dH_2O) for 10 minutes. The same conditions for 1-D SDS-PAGE were also used for the second dimension gel.

Protein transfer: The 2-D SDS-PAGE was soaked in a transfer buffer (48mM Tris base, 39 mM glycine, 20% methanol (MeOH), 1.3 mM SDS) for 15 minutes and placed in a Bio-Rad mini-western Blot apparatus. A PVDF membrane, pre-soaked for 5 seconds in MeOH and rinsed with transfer buffer, was placed in the transfer buffer. The cassette apparatus was set up in the following order, starting from the negative side: sponge, filter, gel, membrane, filter and sponge (making sure no bubbles were present on the membrane). The cassette was placed in the module and a frozen Bio-Ice cooling unit was added. The tank was completely filled with pre-cooled transfer buffer. A stir bar was added to the tank and the entire apparatus was placed in a refrigerator to minimize heating. The power supply was set at 100V for one hour. After transfer, the membrane was rinsed with 100% MeOH and then stained (0.1g coomassie G-250, 40 ml MeOH, 1.0 ml acetic acid and 59 ml ddH_2O stirred for 30 minutes and filtered through a 0.45μ filter) for 60 seconds. The membrane was then de-stained for 2 hours with 50% MeOH and 50% dH_2O.

Protein Sequence: The nitrocellulose membrane, containing the transferred protein, was sequenced by Ken Jackson, Ph.D. at the Molecular Biology Resource Facility, University of Oklahoma Health Science Center (OUHSC).

Basic Local Alignment Search Tool (BLAST) ([23]): A BLASTP was used for protein matching. A BLASTP compares an amino acid query sequence against a protein sequence database. The output from the BLAST search had two critical sets of data: an expected value (e-value) and a score number. An e-value number is equivalent to the number of random hits that occur by chance when searching a database of a given size. The e-value increases exponentially with the score number that is assigned to a match between two sequences. Essentially the e-value described the random background noise that exists for matches between sequences.

Protein Sequence: The nitrocellulose membrane, containing the transferred protein, was sequenced by Ken Jackson, Ph.D. at the Molecular Biology Resource Facility, University of Oklahoma Health Science Center (OUHSC).

Basic Local Alignment Search Tool (BLAST) ([23]): A BLASTP was used for protein matching. A BLASTP compares an amino acid query sequence against a protein sequence database. The output from the BLAST search had two critical sets of data: an expected value (e-value) and a score number. An e-value number is equivalent to the number of random hits that occur by chance when searching a database of a given size. The e-value increases exponentially with the score number that is assigned to a match between two sequences. Essentially the e-value described the random background noise that exists for matches between sequences.

Results

The growth curves for a non-induced and an induced (5 mM phenobarbital) culture of *S. intestinalis* were determined (Fig. 8). Duplicate samples were recorded every 30 minutes for approximately 6 hours.

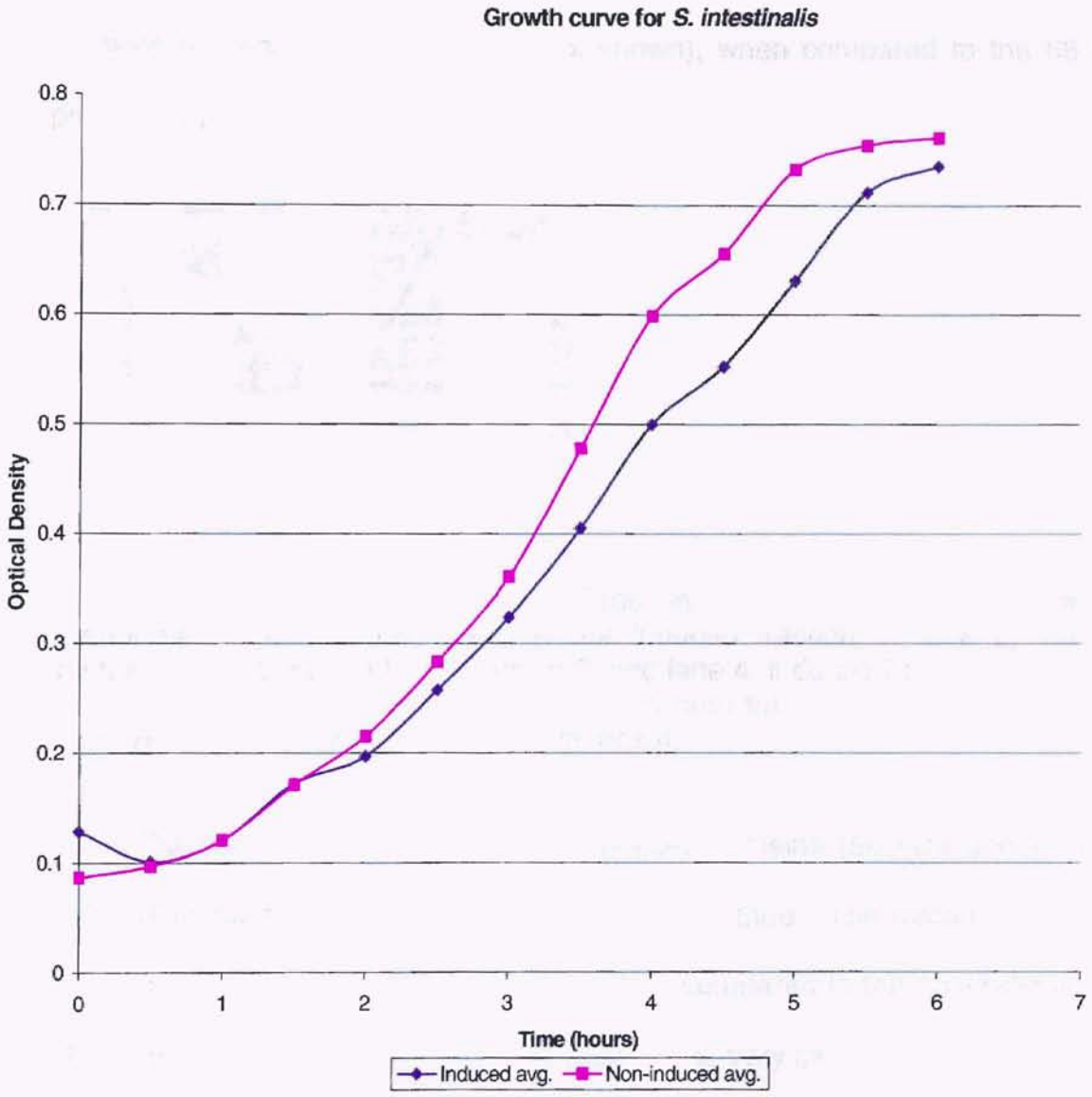

Fig. 8. The growth curve of *S. intestinalis* grown anaerobically at 37°C in BHI media for over 6 hours. The points represent the average of duplicates. The non-induced sample had a doubling time of 0.153 cells/hr and the induced sample had a doubling time of 0.128 cells/hr.

The presence of phenobarbital resulted in no significant difference in the lag, log, or stationary phase for both the non-induced and induced samples (Fig. 8)

The protein profiles for the non-induced and induced cultures were first analyzed by 1-D SDS-PAGE. A 50 kDa and 68 kDa protein were produced in the induced sample (Fig. 9). The expression level for the 50 kDa protein was much higher in both cytoplasmic (fraction 1) and membrane-bound protein (fraction 2) fractions (molecular weight marker not shown), when compared to the 68 kDa protein (Fig.9).

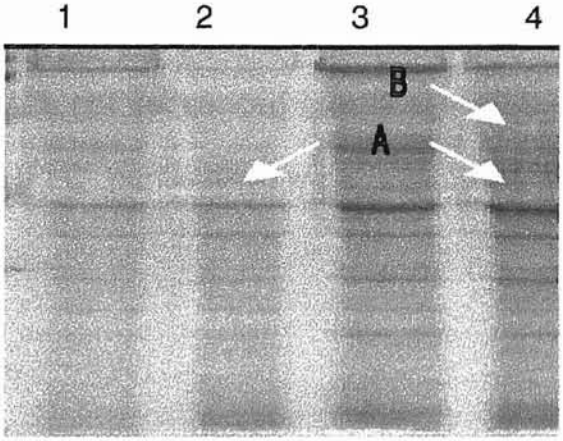

Fig. 9. 1-D SDS-PAGE *S. intestinalis*. (Fraction 1 is cytoplasmic and fraction 2 is membrane-bound proteins) Lane 1, non-induced fraction 1; lane 2, induced fraction 1; lane 3, non-induced fraction 2; and lane 4, induced fraction 2. Arrow A indicates an induced protein near 50 kDa in both fraction 1 and fraction 2 and arrow B an induced protein at 68 kDa in lane 4.

Fraction 2, which contained the induced proteins (50 kDa and 68 kDa) was further purified using the 2-D SDS-PAGE method. The fraction generated proteins at 60 kDa, 50 kDa and 40 kDa, when compared to the non-induced 2-D SDS-PAGE gel. The 68 kDa-induced protein was very close to other proteins on the gel and it was not purified and sequenced. Figure 10 compared the 2-D SDS-PAGE non-induced and induced fraction 2 samples.

The 40 kDa protein (pI 5.0) was purified and sequenced by N-terminal amino acid sequencing. The sequenced results were Gln-Gly-Gln-Gly-Ala-Val-Glu-Gly-Glu-Ile-Asn-Tyr. A BLASTP search with the amino acid sequence revealed a score of 53 and 25.1 bits with an e-value of 111 to protein OprF, a porin protein from *Pseudomonas fuscovaginae*. The 60 kDa protein (pI 6.0) was purified and also sequenced by N-terminal amino acid sequencing. The sequence results were Ala-Ala-Lys-Glu-Val-Lys-Phe-Gly-Asp-Ala-Ala-Arg. A BLASTP search revealed a score of 55 and 25.8 bits with an e-value of 65 to the GroEL, a heat shock protein of *Pseudomonas aeruginosa*. The 50 kDa (pI 7.0) was not sequenced due to a possible N-terminal blockage. Trypsinization and HPLC purification were attempted to prevent N-terminal blockage, but the sequence could not be determined.

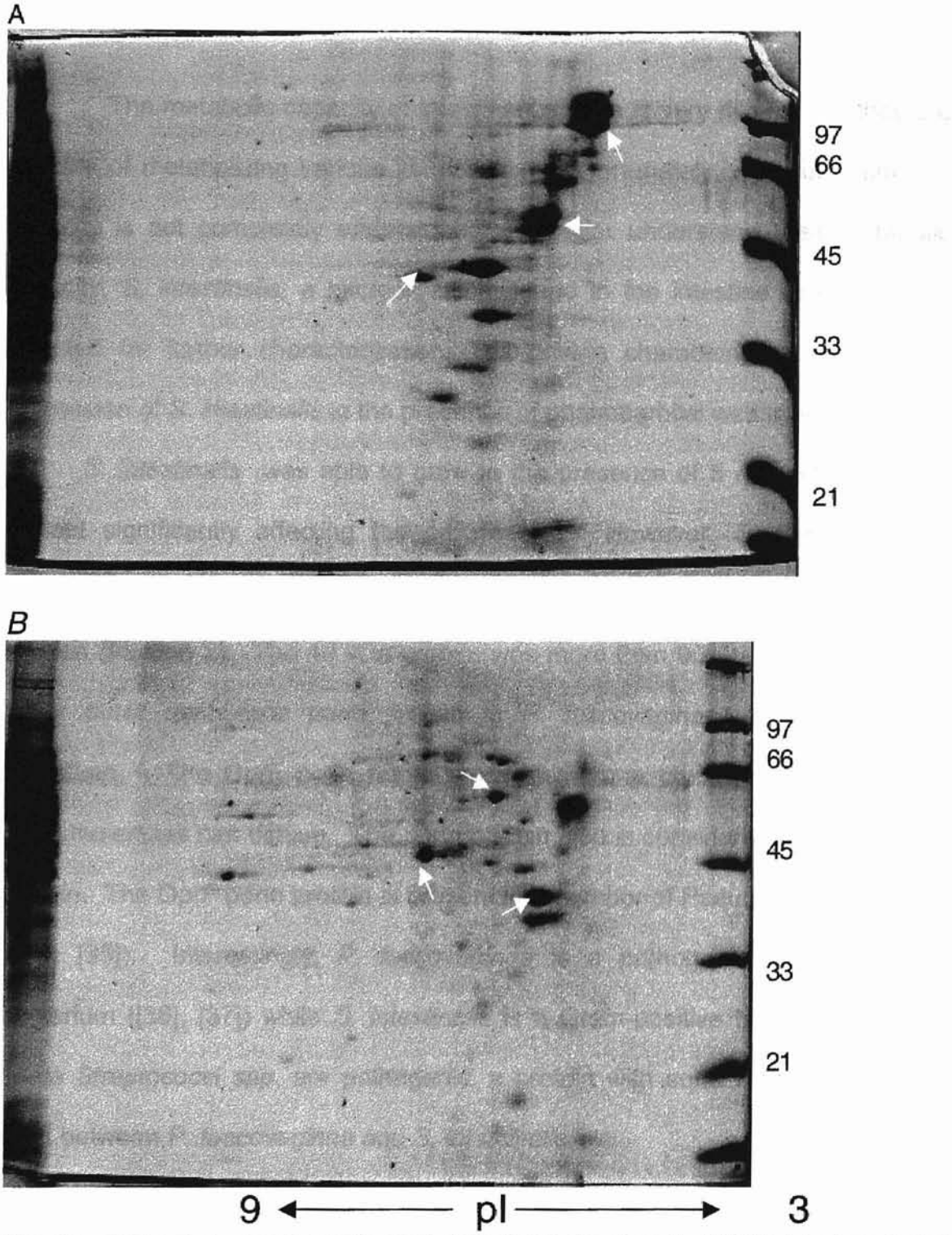

Fig. 10. 2-D gel electrophoresis of *S. intestinalis* fraction 2. (A) Non-induced and (B) induced. The samples were run on a 5% stacking gel and a 12% separating gel. In gel A, arrows show repressed proteins near 42 kDa, 48 kDa, and 97 kDa. In gel B, arrows show induced proteins near molecular weights 60 kDa, 50 kDa and 40 kDa.

Discussion

. The metabolic capacity of intestinal bacteria is very diverse, as they are capable of metabolizing various xenobiotics. Unfortunately, their full metabolic capacity is not completely understood. To better understand their metabolic capacity, *S. intestinalis,* a microorganism found in the intestine of swine, was selected for further characterization. The growth characteristics and protein expression of *S. intestinalis* in the presence of phenobarbital was investigated.

S. intestinalis was able to grow in the presence of 5 mM phenobarbital without significantly affecting the growth rate. However, *S. intestinalis* did produce a number of phenobarbital inducible proteins in the membrane-bound fraction (fraction 2). The 40 kDa protein was more than 90% similar to OprF, a major outer membrane porin protein in *P. fuscovaginae,* a psychrotrophic bacterium. The OprF porin forms cross-membrane channels through which small molecules can diffuse. The porin protein also is considered an attachment protein. The OprF porin protein is present in a number of Pseudomonas species ([34], [35]). Interestingly, *P. fuscovaginae* is a pathogenic, Gram-negative bacterium ([36], [37]) while *S. intestinalis* is a Gram-positive bacterium. Since some *Streptococci spp.* are pathogenic, a protein with conserved regions may exist between *P. fuscovaginae* and *S. intestinalis*.

The 60 kDa protein was sequenced and identified as the GroEL (HSP60) protein, which more than 99% similarity to *P. aeruginosa*, a Gram-negative bacterium. GroEL is a stress protein or a heat shock protein. Since heat shock

proteins are conserved proteins ([38]), it is possible that *S. intestinalis* and *P. fuscovaginae* possess similar proteins.

The results from this study demonstrated that *S. intestinalis* has similar proteins to *P. fuscovaginae* and *P. aeruginosa*. It is unlikely that an outermembrane porin protein is present in *S. intestinalis*, as Gram positive bacteria do not have outer membrane proteins. However, it is possible that a cytoplasmic protein in *S. intestinalis* may resemble the OprF protein. Heat shock protein were originally discovered as cells responded to elevated temperatures. Heat shock proteins are also related to survival of cells under stressful conditions. As a result, heat shock proteins are evolutionarily conserved among bacterial, archaeal, and eukaryotic cells. Therefore, it very possible that *S. intestinalis* may have a similar heat shock protein that resembles the GroEL protein from *P. aeruginosa.* Heat shock proteins are also said to help enhance metabolic systems. Thus, the heat shock protein may be involved in the metabolism of phenobarbital.

Chapter V

Conclusion

The overall goal of this project was to screen intestinal micro-flora for the presence of cytochrome P450. Intestinal bacteria were chosen for the following reasons: 1) they share reductive reactions with cytochrome P450, 2) there is limited data published on the presence of cytochrome P450 in intestinal microflora, and 3) intestinal microflora play a major metabolic role in human physiology.

The methods associated with the project were first developed as they included bacterial DNA preparation, PCR amplification, and sequencing. Based on these developed methods, studies involving the screening of selected intestinal strains for cytochrome P450 were performed.

The following summarizes the overall study: First, five out of eighteen bacterial strains tested, produced an absorbance spectrum when exposed to phenobarbital. No peaks were produced at 450 nm, although two strains produced a peak near the 420 nm mark, suggesting a denatured cytochrome P450. Second, six out of the eighteen strains were positive for an induction of a protein based on the 1-D SDS-PAGE gel. No induced proteins were produced at the predicted size for cytochrome P450 (45-55 kDa and 119 kDa). Of these six strains, *B. infantis* and *S. intestinalis* produced a spectrum based on exposure to phenobarbital. Third, *S. intestinalis* was further studied as it was selected based on the fact that the induced protein bands from the 1D SDS-PAGE gel were

clearly separate from other non-induced proteins. The induced proteins from *S. intestinalis* were subsequently sequenced. The sequenced information determined that the proteins were a porin protein and a heat shock protein.

Based on the lack of a specific peak at 450 nm or 420 nm and no phenobarbital induction at the predicted size range (45 kDa and 116 kDa), it appears that the selected strains of intestinal bacteria from the human and the single swine isolates do not contain cytochrome P450. In addition, sequence information did not match with existing cytochrome P450 families. It should be noted that other minor induced proteins from the other bacterial species were characterized. The characterization of those proteins may provide some interesting data regarding the response these bacterial species had against phenobarbital.

The hunt for P450s in intestinal bacteria may involve using a different inducer. Finally, the complete genome sequencing of major intestinal flora will provide information regarding the presence of a cytochrome P450 or a P450-like protein. Therefore, the work associated with finding the a cytochrome P450 in intestinal bacteria is far from over.

Recently, the bacterium *Eubacterium aerofacians* was shown to possess a cytochrome P450-like protein ([39]).

Chapter VI

References

1. Gonzalez, F.J. and D.W. Nebert, *Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation.* 1990. **6**(6): p. 182-186.
2. Nelson, D.R., *et al.*, *The P450 Superfamily: Update on New Sequences, Gene Mappin, Accession Numbers, Early Trivial Names of Enzymes and Nomenclature.* DNA and Cell Biology, 1993. **12**(1): p. 1-51.
3. Nelson, D.R., *Cytochrome P450 and the Individuality of Species.* Archives of Biochemistry and Biophysics, 1999. **369**(1): p. 1-10.
4. Geoptar, A.R., H. Scheerens, and N.P.E. Vermeulen, *Oxygen and Xenobiotic Reductase Activities of Cytochrome P450.* Critical Reviews in Toxicology, 1995. **25**(1): p. 25-65.
5. Klingenberg, M., *Pigments of Rat Liver Microsomes.* Archives of Biochemistry and Biophysics, 1958. **75**: p. 376-386.
6. Garfinkel, D., *Studies on Pig Liver Microsomes. I. Enzymatic and Pigment Composition of Different Microsomal Fractions.* Archives of Biochemistry and Biophysics, 1958. **77**: p. 493-509.
7. Omura, T. and R. Sato, *The Carbon Monoxide-binding Pigment of Liver Microsomes I. Evidence for its hemoprotein Nature.* The Journal of Biological Chemistry, 1964. **239**(7): p. 2730-2378.
8. Omura, T. and R. Sato, *The Carbon Monoxide-binding Pigment of Liver Microsomes II. Solubiliztion, Purification, and Properties.* Journal of Biological Chemistry, 1964. **239**(7): p. 2379-2385.
9. Katagiri, M., B.N. Ganguli, and I.C. Gunsalus, *A Soluble Cytochrome P-450 Functional in Methylene Hydroxylation.* Journal of Biological Chemistry, 1968. **243**(12): p. 3543-3546.
10. Poulos, T.L., *et al.*, *The crystal structure of cytochrome P450cam.* Journal of Biological Chemistry, 1985. **260**: p. 16122-16130.
11. Black, S.D. and M.J. Coon, *P-450 Cytochromes: Structure and Function.* Advanced Enzymology, 1987. **60**: p. 35-87.
12. Fulco, A.J., *$P450_{BM-3}$ and other Inducible Bacterial P450 Cytochromes: Biochemistry and Regulation.* Annual Review of Pharmacology and Toxicology, 1991. **31**: p. 177-203.
13. Patel, N.V. and C.A. Omer, *Phenobarbital and sulfonylurea-inducible operons incoding herbicide metabolizing cytochromes P-450 in Streptomyces griseolus.* Gene, 1992. **112**: p. 67-76.
14. Nelson, D.R., *et al.*, *P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature.* Pharmacogenetics, 1996. **6**: p. 1-42.

15. Waxman, D.J., *P450 gene induction by structurally diverse xenochemicals: Central role of Nuclear receptors CAR, PXR and PPAR.* Archives of Biochemistry and Biophysics, 1999. **369**(1): p. 11-23.
16. Graham, S.E. and J.A. Peterson, *How similar are P450s and what can their differences teach us?* Archives of Biochemistry and Biophysics, 1999. **369**(1): p. 24-29.
17. Goldin, B., *Intestinal Microflora: Metabolism of Drugs and Carcinogens.* Annals of Medicine, 1990. **22**: p. 43-48.
18. Rowland, I.R., *Interactions of the Gut Microflora and the Host in Toxicology.* Toxicologic Pathology, 1988. **16**(2): p. 147-152.
19. Bokkenheuser, V., *The Friendly Anaerobes.* Clinical Infectious Diseases, 1993. **16**(Sup. 4): p. S427-34.
20. Falk, P.G., *et al.*, *Creating and Maintaining the Gastrointestinal Ecosystem: What we Know and Need to Know from Gnotobiology.* Microbiology and molecular Biology Reviews, 1998. **62**(4): p. 1157-1170.
21. Rumney, C.J. and I.R. Rowland, *In Vivo and In Vitro Models of the Human Colonic Flora.* Critical Reviews in Food Science and Nutrition, 1992. **31**(4): p. 32.
22. Smith, R.V., *Metabolism of Drugs and Other Foreign Compounds by Intestinal Microorganisms.* Wld Rev. Nutr. Diet, 1978. **29**: p. 60-76.
23. Altschul, S., *et al.*, *Basic local alignment search tool.* Journal of Molecular Biology, 1990. **215**(3): p. 402-410.
24. Snyder, M.J., *et al.*, *Sampling P450 Diversity by Cloning Polymerase Chain Reaction Products Obtained with Degenerate Primers.* Methods in Enzymology, 1996. **272**: p. 304-312.
25. Moore, W.E.C. and L.V. Holdeman, *Human Fecal Flora: The Normal Flora of 20 Japanese-Hawaiians.* Applied Microbiology, 1974. **27**(5): p. 961-979.
26. Ingle, C.A. and S.R. Kushner, *Development of an in vitro mRNA decay system for Escherichia coli: Poly (A) polymerase I is necessary to trigger degradation.* Procedures of the National Academics of Science, 1996. **93**: p. 12926-12931.
27. Munro, A.W. and J.G. Lindsay, *Bacterial cytochromes P-450.* Molecular Microbiology, 1996. **20**(6): p. 1115-1125.
28. Blattner, F.R., *et al.*, *The Complete Genome Sequence of Escherichia coli K-12.* Science, 1997. **277**: p. 1453-1462.
29. John, G.H., *et al.*, *Escherichia coli expression and characterization of cytochromes P450 2I and 2B5.* Archives of Biochemistry and Biophysics, 1994. **314**(2): p. 367-375.
30. Hill, M.J., *Intestinal flora and endogenous vitamin synthesis.* European Journal of Cancer Prevention, 1997. **6**(Sup 1): p. S43-S45.
31. Robinson, I.M., *et al.*, *Streptococcus intestinalis, a New Species from the Colons and Feces of Pigs.* International Journal of Systematic Bacteriology, 1988. **38**(3): p. 245-248.
32. Vandamme, P., *et al.*, *Streptococcus intestinalis Robinson et al. 1988 and Streptococcus alactolyticus Farrow et al. 1984 are phenotypically*

indistinguishable. International Journal of Systematic Bacteriology, 1999. **49**: p. 737-741.
33. Nugon-Baudon, L., *et al.*, *Effect of the bacterial status of rats on the changes in some liver cytochrome P450 (EC 1.14.14.1) apoproteins consequent to a glucosinolate-rich diet.* British Journal of Nutrition, 1998. **80**(3): p. 231-234.
34. Delihas, N., *Antisense micF RNA and 5`-UTR of the target ompF RNA: phylogenetic conservation of primary and secondary structures.* Nucleic acids symposium series, 1997. **36**: p. 33-35.
35. Lomas, J.G.d., D. Navarro, and C. Gimeno, *Penetration of meropenem in gram-negative bacilli. Differences in activity with imipenem.* Enfermedades infecciosas y microbiologia clinica, 1997. **15**(Suppl 1): p. 14-19.
36. Hu, F., J. Young, and M. Fletcher, *Preliminary description of biocidal (syringomycin) activity in florescent plant pathogenic Pseudomonas species.* Journal of Applied Microbiology, 1998. **85**(2): p. 365-371.
37. Bare, S., *et al.*, *Conformations in solution of the fuscopeptins Phytotoxic metabolites of Pseudomonas fuscovaginae.* European Journal of Biochemistry, 1999. **266**(2): p. 484-492.
38. Brocchieri, L. and S. Karlin, *Conservation among HSP60 sequences in relation to structure, function, and evolution.* Protein Science, 2000. **9**(3): p. 476-486.
39. John, G.H., *et al.*, *The Presence of a Cytochrome P450-like Protein in the Human Intestinal Microflora Eubacterium aerofaciens.* Microbial Ecology in Health and Disease, 2001. **13**: p. 3-8.

VITA

Shannon D. Walls

Candidate for the Degree of

Master of Science

Thesis: THE SCREENING OF INTESTINAL BACTERIA FOR THE PRESENCE OF CYTOCHROME P450 WITH INDUCTION BY PHENOBARBITAL

Major Field: Microbiology, Cell, and Molecular Biology

Biographical:

Education: Graduated from Norman High School, Norman, Oklahoma in May 1989; received Bachelor of Science degree in Microbiology and a Bachelor of Science degree in Molecular Genetics from Oklahoma State University, Stillwater, Oklahoma in December 1995. Completed the requirements for the Master of Science degree with a major in Microbiology, Cell, and Molecular Biology at Oklahoma State University, Department of Microbiology and Molecular Genetics in December 2001.

Experience: Employed by Oklahoma State University, Department of Microbiology and Molecular Genetics as an undergraduate and as a graduate research assistant and teaching assistant, 1994 to 2000. Employed by University of Nebraska Medical Center as a Research Technologist II, 2000 to present.

Professional Memberships: American Society of Microbiology