THE METABOLISM OF IBUPROFEN BY SPHINGOMONAS IBU-2

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Pharmaceutical have been detected in bodies of water all over the world and their environmental fates are the subject of increasing scientific attention. *Sphingomonas* Ibu-2 was isolated from a wastewater treatment plant based on its ability to use ibuprofen as a sole carbon and energy source. A yellow color indicative of meta-cleavage accumulated in the culture supernatant when Ibu-2 was grown on ibuprofen. When 3-flurocatechol was used to poison the meta-cleavage system, isobutylcatechol was identified in the culture supernatant via GC/MS analysis. Ibuprofen-induced washed cell suspension also metabolized phenylacetic acid to catechol. A chromosomal library of Ibu-2 was generated in *Escherichia coli* and yielded a single fosmid clone (*E. coli* epi300 pFOS3G7) capable of metabolizing ibuprofen to isobutylcatechol. Transposon mutagenesis of pFOS3G7 and screening for loss of function revealed insertions in five open reading frames *ipfABDEF* whose predicted amino acid sequences bore similarity to the large and small units of aromatic dioxygenases, an SCPx thiolase, domain of unknown function 35 (DUF35), and an aromatic coenzyme A ligase, respectively. Complementation of the *E. coli* epi300 pFOS3G7 ipfD deletion mutant restored catechol-production. An *E. coli* subclone expressing *ipfABDEF* from pFOS3G7 produced trace catecholic metabolites from phenylacetic acid derivatives, suggesting that these genes were sufficient for the observed activity. The co-expression of *ipfABDEF* with *ipfH* and

ipfI, a ferredoxin reductase and a ferredoxin respectively which were both identified on pFOS3G7, greatly enhanced the production of catecholic metabolites. Further analysis revealed that cell-free extracts of *E. coli* harboring a high copy number *ipfF* expression vector catalyzed the ATP and magnesium-dependent ligation of coenzyme A to both phenyacetate and ibuprofen. Uptake of phenylacetic acid was also found to be driven by the ibuprofen coenzyme A ligase IpfF; when *ipfF* was knocked out, uptake was decreased to negative control levels as determined by radiolabelled phenyletate uptake assays. These results are consistent with ibuprofen uptake occurring via a vectorial acylation mechanism. Taken toghether, the results described in this dissertation demonstrate that in contrast to the widely distributed coenzyme A ligase, homogentisate, or homoprotocatechuate pathways for metabolism of phenylacetic acids, Ibu-2 removes the acidic side chain of ibuprofen and related compounds prior to ring-cleavage. The involvement of a novel SCPx thiolase (IpfD) and a DUF35 protein (IpfE) in the generation of the catechol suggest that this pathway is distinct from others described to date and represents a new aerobic paradigm for the metabolism of phenylacetic acids

BIOGRAPHICAL SKETCH

Robert was raised near Houston where he spent his childhood exploring the bayous and piney woods of East Texas. He had the honor of attending the Texas Academy of Math and Science north of Dallas, where he simultaneously completed two years of high school and two years of college courses at the University of North Texas. Robert then attended the University of Texas at Austin where he earned his Bachelor's degree in Zoology. For the following two years, Robert worked as a fisheries biologist for the National Marine Fisheries Service. The majority of this time was spent on board commercial deep sea fishing vessels. He earned his sea legs on the Bering Sea, the North Pacific off the coast of Washington, and the Pacific Ocean south of Hawaii, racking up a total of ten months worth of days at sea. Robert then entered the Cornell University Environmental Toxicology PhD program. In addition to diverse coursework in risk studies, toxicology, and microbiology, Robert learned the molecular biology and biodegradation ropes under the mentorship of Dr. Anthony Hay. After five years, Robert took a job with Dr. Andria Costello at Syracuse University for two years studying environmental microbiology, after which he returned to Cornell University to complete his PhD dissertation.

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LIST OF ABBREVIATIONS AND SYMBOLS

2,4-DNP: 2,4 dinitrophenol
2PPA: 2-phenylpropionic acid
3FC: 3-fluorocatechol
3TAA: 3-tolylacetic acid
4TAA: 4-tolylacetic acid
ABC: ATP binding cassette
ADP: adenosine diphosphate
Amp: ampicillin
AntDO- anthranilate 1,2-dioxygenase
ATP: adenosine triphosphate
BMO: bacterial monooxygenase
CCIS: copy control induction solution
CDD: conserved domain database
CE: capillary electrophoresis
Chl: chloramphenicol
CoA: coenzyme A
COX: cyclooxygenase
DNA: deoxyribonucleic acid
DTT: dithiothreitol
DUF35: domain of unknown function 35
EF: enantiomeric fraction
FeDox: ferredoxin
GC/MS: gas chromatography mass spectroscopy
GSH: glutathione
HPLC: high performance liquid chromatography
IPF: ibuprofen
IPTG: Isopropyl β-D-1-thiogalactopyranoside
Kan: kanamycin
K_{ow}: octanol water partitioning coefficient
LB: Luria-Bertani broth
LOEC: lowest observed effects concentration
MFS: major facilitator superfamily
mM: milimolar
MSM: mineral salts medium
NAD+: oxidized nicotinamide adenine dinucleotide
NADH: nicotinamide adenine dinucleotide
NADP+: oxidized nicotinamide adenine dinucleotide phosphate
NADPH: nicotinamide adenine dinucleotide phosphate
NCBI: National Center for Biotechnology Information
NIH-NIEHS: National Institutes of Health – National Institute of Environmental Health Sciences
NSAID: nonsteroidal anti-inflammatory
OB-fold: oligosaccharide/oligonucleotide binding fold

OD_{600}: optical density at 600 nanometers
ORF: open reading frame
PAA: phenylacetic acid
PCR: polymerase chain reaction
PMSF: phenylmethylsulfonyl fluoride
Ppb: part per billion
Ppm: part per million
RBS: ribosomal binding site
RHO: Rieske-type non-heme iron aromatic ring-hydroxylating oxygenases
RT-PCR: reverse transcriptase PCR
SCP-2: sterol carrier protein 2
SCPx: sterol carrier protein X
SOE: strand overlap extension
STP: sewage treatment plant
TCA: tricarboxylic acid cycle
Tet: tetracycline
μM: micromolar
WCS: washed cell suspension
X-gal: bromo-chloro-indolyl-galactopyranoside
Δ: gene deletion mutant

CHAPTER 1

INTRODUCTION

Pharmaceuticals in the Environment

The ecotoxicology community has become increasingly concerned with the issue of human pharmaceuticals as potential environmental contaminants (Halling Sorensen, Nielsen et al. 1998; Daughton and Ternes 1999; Jorgensen and Halling-Sorensen 2000; Dietrich, Webb et al. 2002, Kagle et al. 2009). We have become distinctly aware that the world is not a limitless sink for our wastes (Andonova and Mitchell 2010; Peattie 2010; Schwarzenbach, Egli et al. 2010). At this stage in our cultural evolution, we are quite aware that our waste products can adversely impact the natural environment and ourselves.

Within the last decade, an initially puzzling and cataclysmic drop in Central Asian vulture populations was clearly linked to the veterinary use of the anti-inflammatory diclofenac (Oaks, Gilbert et al. 2004). These species of vultures were powerfully impacted due to a combination of very high exposure levels via feeding on deceased, recently-dosed cattle and an extremely severe idiosyncratic nephritic sensitivity that led to death by visceral gout (Oaks, Gilbert et al. 2004). The severe consequences of this entirely unanticipated toxic manifestation will likely serve as a rallying cry against the indiscriminant use of pharmaceuticals in the environment for a long time to come.

While on one hand it can be argued that virtually no adverse environmental effect has ever been linked to the environmental presence of human (rather than veterinary)

pharmaceuticals, that pharmaceuticals are metabolized by the human body and are mobile and readily diluted in the hydrosphere such that they will not reach notable concentrations (Jones, Voulvoulis et al. 2004). On the other hand, unlike many pollutants traditionally given much more attention, pharmaceuticals are specifically designed to be biologically active. Even though they are released in low concentrations, they are released in an overwhelmingly complex diversity of parent compounds and metabolites, raising the possibility of addition and synergistic toxicity (Flaherty and Dodson 2005; Chou 2006; Laetz, Baldwin et al. 2009). Mixture toxicity is a poorly developed, highly controversial field (Chou 2006; Baas, van Houte et al. 2007). Predictions of mixture effects are currently not feasible due in large part to the absence of precise dose-relevant mechanistic data (Borgert, Quill et al. 2004; Cedergreen, Christensen et al. 2008; Crofton 2008; Kortenkamp 2008).

Given the inadequacy of the knowledge base regarding the potential effects of these compounds at environmentally relevant concentrations, many governments the world over have begun to apply the loosely-defined Precautionary Principle to guide policy development in regards to environmental risks (Ferrari and Mons et al. 2004; Gardiner 2006). Whereas classical risk analysis and cost-benefit analysis depend upon having a handle on the mathematical underpinnings of cause-effect relationships, the Precautionary Principle is invoked in the absence of proof of causation (Raffensberger and Tickner 1999; Gardiner 2006). Given the complexity of the problem, the subtlety of the potential effects involved, and the limited scientific information available, it seems that application of the conservative Precautionary Principle may be the best way to limit the potential environmental risks of pharmaceuticals.

Pharmaceuticals are released primarily from sewage treatment plants (STPs) into bodies of water. There is potential for untreated waste to be released when STP capacity is exceeded or for effluent to be poorly diluted during periods of drought, during which STP effluent may be the main source of flow, thus leading to concentration spikes (Loraine and Pettigrove 2006). For example, concentrations of pharmaceuticals in STP effluent can vary by over an order of magnitude depending on rainfall levels (Peng, Yu et al. 2008). Additionally, while most pharmaceuticals investigated so far have are effectively removed during wastewater treatment (>90%), many, such as carbemezipine, clofibric acid, and diclofenac, are not (Heberer 2002). Additionally, little work has been done to resolve what amount of apparent removal might be due to sorption to the solid phase followed by land application of the resulting solids (Xia, Bhandari et al. 2005). Ternes *et al.* suggest that solid phase sorption is likely a very significant fate for pharmaceuticals depending on their charge and K_{ow} (Ternes, Joss et al. 2005). In the few studies that have investigated the capacity of acidic pharmaceuticals to sorb to solid phases, little or no sorbtion capacity has been observed (Heberer, Verstraeten et al. 2001; Jones, Lester et al. 2005), though these studies have not considered biolsolids in particular. This route is generally regarded as more important for lipophiles or for chemicals that have the capacity to sorb to the sludge via other mechanisms (Rabiet, Togola et al. 2006).

Release and dilution into the hydrosphere is not the only fate of treated wastewater. Treated wastewater is being increasingly used as irrigation water, particularly in dry western states in the USA (Pedersen, Yeager et al. 2003; Pedersen, Soliman et al. 2005; Kinney, Furlong et al. 2006). Kinney *et al.* (Kinney, Furlong et al.

2006) state that "in 1993, it was estimated that 2.4% of wastewater effluent was used for irrigation in the United States." Only a decade later, it was estimated that the percentage had increased to 7.4% (Xu, Wu et al. 2009). In especially dry, densely populated areas such as Israel, the percentage of reclaimed water used for irrigation can be as much as 66% (Kinney, Furlong et al. 2006). The xenobiotics remaining in this treated wastewater are available to directly impact soil ecosystems with little or no dilution. Indeed, common pharmaceuticals have been detected at part-per-billion levels in both treated wastewater used for irrigation and associated agricultural fields in Colorado (Kinney, Furlong et al. 2006), Mexico City (Siemens, Huschek et al. 2008), and southern California (Xu, Wu et al. 2009) and were even found to persist from one growing season to another (Kinney, Furlong et al. 2006). As populations and urbanization increase, this will become a more and more prevalent practice.

Of potentially greater importance from a political and public perception viewpoint, is the possibility of contamination of drinking water with pharmaceuticals. Chefetz *et al.* (Chefetz, Mualem et al. 2008) showed that several common pharmaceuticals are readily mobile in soil and may reach groundwater following application to agricultural fields via sludge or treated wastewater, establishing a mode of transfer between the human waste stream and water reservoirs. A handful of studies have examined to what extent drinking water treatment methods are effective at removal of pharmaceutical residues. An investigation by Stackelberg *et al.* (Stackelberg, Gibs et al. 2007) found that the most common methods such as chlorination and charcoal filtration are partially effective, removing some compounds, but not others, though the authors point out that chlorination could be generating an array of unknown chemically activated

reaction products. Huerta-Fontella and Fontella (Huerta-Fontela and Ventura 2008) showed that a more thorough drinking water treatment process that includes several oxidative and advanced filtration steps is almost completely effective at removing pharmaceutical residues.

Presumably due to constant release into the hydrosphere via wastewater effluent processes, direct land application followed by transport through groundwater systems, and incomplete removal during drinking water treatment processes, a very wide variety of pharmaceuticals have been detected in drinking water supplies at part-per-trillion to part-per-billion levels the world over (see (Fent, Weston et al. 2006; Benotti, Trenholm et al. 2008; Huerta-Fontela and Ventura 2008; Corcoran, Winter et al. 2010; Kummerer 2010) for summaries of available data). However, a recent thorough risk analysis of fifteen commonly detected pharmaceuticals with known endpoints including carcinogenic, developmental, and reproductive toxicity predicted no effects on humans at any concentrations that have been detected in drinking water (Bruce, Pleus et al. 2010). A thorough review of available pharmaceutical ecotoxicological data by Fent *et al.* (Fent, Weston et al. 2006) showed that with few exceptions, available lowest observed effect concentration (LOEC) data suggests virtually no environmental impact of such compounds, though they point out that such data is sorely lacking for individual compounds and almost nonexistent for complex mixtures. Efforts to create a comprehensive database of pharmaceutical ecotoxicological data are currently underway (www.wikipharma.org (Molander, Agerstrand et al. 2009)).

The scientific and policy communities are faced with a vastly complex issue where the potential for harm is not understood due to a lack of data regarding the

toxicological and ecological questions posed. Several comprehensive reviews are available that summarize data regarding the presence and effects of pharmaceuticals. They all come to the same conclusion: that not enough data exists to adequately answer the pertinent questions (Stuer-Lauridsen, Birkved et al. 2000; Heberer 2002; Jones, Voulvoulis et al. 2004; Cunningham, Buzby et al. 2006; DeLange, Noordoven et al. 2006; Fent, Weston et al. 2006; Hernando, Mezcua et al. 2006; Schwarzenbach, Escher et al. 2006; Dorne, Skinner et al. 2007; Kummerer 2008; Kummerer 2010; Santos, Araújo et al. 2010).

Ibuprofen

Ibuprofen is one of the most commonly consumed pharmaceuticals in the world (see (Fent, Weston et al. 2006) for a partial review of country-by-country consumption). It is also a good candidate for study because it is a member of a diverse class of compounds that share certain structural features in common: the non-steroidal anti-inflammatories (NSAIDs). The NSAIDs themselves are a very significant class of pharmaceuticals from an environmental risk perspective. The dose of most NSAIDs is particularly high, typically hundreds of milligrams to grams per day. Many NSAIDs are purchased over the counter with virtually no regulation, largely because of their very broad margin of safety; for example, ibuprofen is considered one of the safest drugs ever (Rainsford 2009). There are also many NSAIDs that are available by prescription, either because they serve specific needs or because they are young in a regulatory sense. Many of these NSAIDs share a phenylacetic acid moiety which is believed to be responsible for

their activity (Figure 1.1).

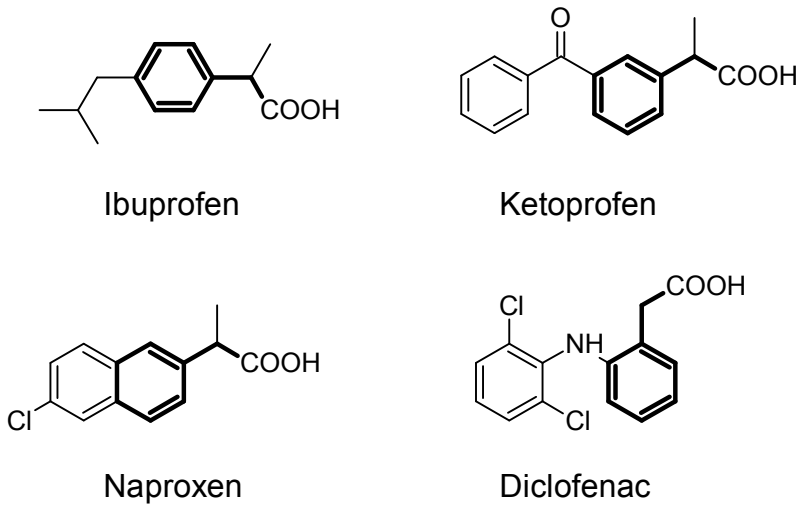

Figure 1.1. Common non-steroidal anti-inflammatory pharmaceuticals with a phenylacetic acid moiety (bolded).

Their typical mode of action is the inhibition of cyclo-oxygenase (COX) enzymes that cleave arachadonic acid in humans to create prostglandins which are potent paracrine signaling compounds (Figure 1.2) (Fent, Weston et al. 2006).

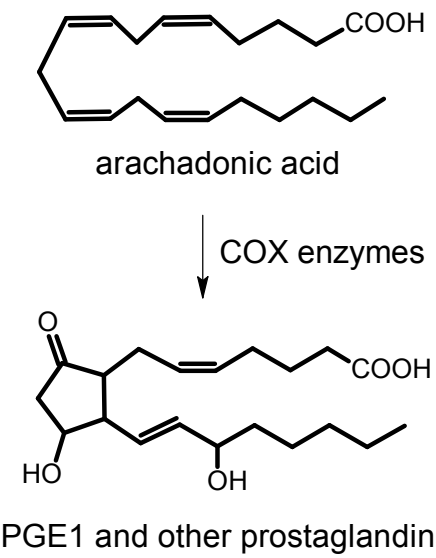

Figure 1.2. General action of cyclooxygenase enzymes (Fent, Weston et al. 2006).

However, prostglandins are a rather broad class of paracrine compounds that play a variety of roles in animals in addition to inflammation including regulation of gastric mucosa (Vane 1971) renal vascular tissue (Hao and Breyer 2008) and egg shell thickness in birds (Lundholm 1997). Prostglandins also play many roles in fish including control of ovulation (Mercure and Van der Kraak 1996; Sorbera, Asturiano et al. 2001), sex hormone regulation (Lister and Van der Kraak 2008), sexual behavior (Sorensen and Goetz 1993), cortisol regulation (Mommsen, Vijayan et al. 1999) and even function as sex pheromones (Sorensen and Geotz 1988; Moore and Waring 1996). At high doses, NSAIDs can cause liver damage following metabolic activation (reviewed by Fent *et al.* (Fent, Weston et al. 2006)), although the relevance of this toxic outcome at representative environmental concentrations is unknown.

In addition to its wide consumption and structural similarity to a number of other pharmaceuticals, an obvious reason to investigate the fate of ibuprofen is the fact that

ibuprofen has been detected in bodies of water all over the world. Environmental concentrations of ibuprofen have been found to range from low part-per-trillion (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002) to low part-per-billion levels (Buser, Poiger et al. 1999; Farre, Ferrer et al. 2001) (See (Santos, Araújo et al. 2010) for a thorough listing of available environmental concentration data). Ibuprofen has been found in trace concentrations in such disparate locations as drinking water (Heberer, Verstraeten et al. 2001; Jones, Lester et al. 2005; Loraine and Pettigrove 2006; Rabiet, Togola et al. 2006) and the middle of the North Sea (Buser and D. 1998).

The fate of ibuprofen in the environment

The primary pathway by which ibuprofen enters the environment is believed to be via human waste and sewage treatment plants, though deposition into landfills is a poorly-understood alternate route (Bound and Voulvoulis 2005). A significant amount of ibuprofen consumed by humans, 8.9% - 14%, is excreted unmodified or as the glucuronide conjugate (Rudy, Knight et al. 1991; Lee, Peart et al. 2005). The primary human metabolite of ibuprofen is hydroxyibuprofen (Figure 1.3), which has been detected in sewage treatment plants but whose fate and effects are otherwise completely unknown (Paxeus 2004). Two other minor human ibuprofen metabolites carboxyibuprofen and carboxyhydratropic acid (Figure 3) are similarly uninvestigated.

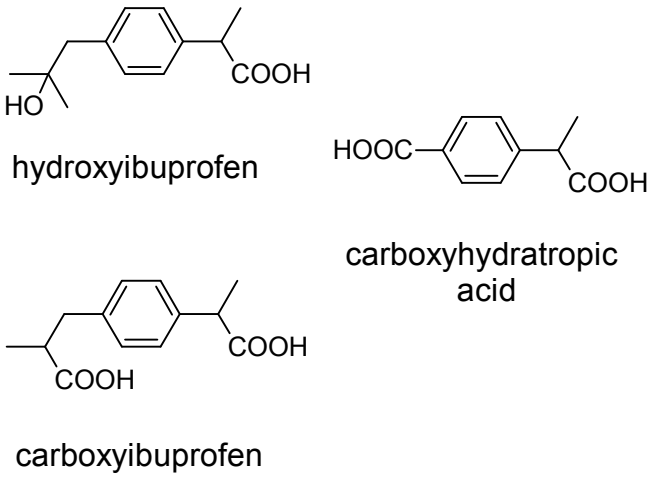

Figure 1.3. Human primary metabolites of ibuprofen (Rudy, Knight et al. 1991; Lee, Peart et al. 2005).

Given all the pathways by which ibuprofen can enter the environment combined with the high therapeutic dose and high rate of consumption, it is not surprising that ibuprofen is one of the most frequently detected pharmaceutical compounds in aquatic systems. The input, while somewhat sporadic, is continuous for the most part, leading to a situation of continual low level exposure for the exposed aquatic communities. Hydroxyibuprofen, the primary human metabolite of ibuprofen, has also been detected though not quantified in surface waters (Winkler, Lawrence et al. 2001).

As soon as ibuprofen enters the waste stream, it is likely to be acted upon by microorganisms via two general types of metabolic/enzymatic systems: non-specific co-metabolic processes or assimilative metabolism. The biological transformation of ibuprofen has been investigated in broad strokes using analytical chemistry techniques. Microbe-dependent accumulation of hydroxyibuprofen and carboxyibuprofen (identical to those illustrated in Figure 1.3) have been observed in a handful of studies (Zwiener, Seeger et al. 2002; Quintana, Weiss et al. 2005; Marco-Urrea, Pérez-Trujillo et al. 2009),

though in each those studies these the side-chain oxidation products that accumulated only accounted for a small amount of ibuprofen disappearance. There is no evidence in the literature that the assimilative metabolism of ibuprofen has been investigated outside of the work presented in this dissertation.

Ibuprofen has been found to be biologically degraded in sewage treatment plants and in the aquatic environment with varying efficiency. Buser *et al.* (Buser, Poiger et al. 1999) found ibuprofen to have a half-life of 3 hours in activated sludge and 20 days in lake water. Zweiner *et al.* (Zwiener, Seeger et al. 2002) found ibuprofen to have a half-life of less than a day in a batch experiment. Sewage treatment plants have for the most part been shown to be at least 90-95% efficient in removal of ibuprofen (96%-99.9%; (Buser, Poiger et al. 1999)) with certain exceptions (22%-75%; (Stumpf, Ternes et al. 1999)), particularly in the developing world where STP performance can be highly variable. Whether the ibuprofen is mineralized (degraded all the way to carbon dioxide and water), transformed to stable but as of yet unidentified products, assimilated into biomass, or sorbed by solids, etc. has not been investigated. Buser *et al.* (Buser, Poiger et al. 1999) did demonstrate, however, that ibuprofen is rather persistent in sterile lake-water, with no degradation occurring within 37 days even when exposed to daylight. An investigation in to the persistence of ibuprofen in a low-nutrient Swiss lake-water found that ibuprofen was surprisingly recalcitrant, with a half-life of 32 days (Tixier, Singer et al. 2003). In this system, ibuprofen did not sorb to sediment to a significant degree, nor did it volatilize.

Ibuprofen is non-volatile (Henry's Law constant = 1.23×10^{-6} atm m^3/mole), sparingly water soluble (Kow = 3.97 (Jones, Lester et al. 2005)), does not sorb to

sediment (Jones, Lester et al. 2005), and is little-affected by physical degradative forces. These characteristics are similar to many other pharmaceuticals (Tixier, Singer et al. 2003) and can be said to be an adequate, if crude, picture for the fate of pharmaceuticals in general. In fact, pharmaceuticals are designed specifically to have these properties: they must be non-volatile and physically stable so that they have a respectable shelf-life.

The fact that ibuprofen is chiral should not be excluded from studies on ibuprofen fate. The formulation of ibuprofen in use across the world is a 50/50 R/S mixture with the exception of some specialty products in use in Europe. This is likely because no method for isolating the active enantiomer is sufficiently cost-effective to justify the marginal benefits provided. The S-enantiomer is the biologically active compound. The R-enantiomer has no known biological activity. Humans readily perform a chiral inversion on ibuprofen, converting the R- to the S-form (Daughton 2002). Therefore, ibuprofen and its byproducts are primarily excreted as the S-enantiomer. Interestingly, Buser *et al.* (Buser, Poiger et al. 1999) found that the R isomer persisted longer in biologically active lake water spiked with racemic ibuprofen, implying either that a chiral inversion mechanism (S to R) was at work and/or that the S-enantiomer is more readily degraded.

While, as mentioned above, some STPs remove up to 99% of the ibuprofen load (Buser, Poiger et al. 1999), these facilities still represent of a source from which ibuprofen can reach the environment. Simply due to the constancy of their outputs, many STPs must also occasionally release untreated waste during periods of high precipitation or equipment failure (Loraine and Pettigrove 2006). Also, during dry periods, the bodies of water that sewage effluent is discharged into can be depleted, thus limiting the effect

of dilution and thereby effectively increasing the concentration of ibuprofen in the receiving waters (Loraine and Pettigrove 2006).

The fate of unused pharmaceuticals is largely unknown. One survey-based British research project was conducted so as to gain some insight on this subject for ibuprofen and three other pharmaceuticals (Bound and Voulvoulis 2005). They might be flushed down the toilet, in which case they would enter the same waste stream as outlined above. Alternatively, people may return them to a pharmacy, a route that was found to be rather uncommon in the case of ibuprofen. The primary mode of ibuprofen disposal was found to be via solid waste disposal, which overall accounted for 8% of total ibuprofen fate in the Bound *et al.* study (Bound and Voulvoulis 2005).

Ibuprofen Ecotoxicology

A comprehensive body of literature exists that describes the toxicology of ibuprofen in mammalian systems at or near concentrations reached in the body during normal therapeutic use, which are far above what is ever seen in the environment (reviewed in (Rainsford 1999)). Ibuprofen has also been found to inhibit the growth of common pathogenic bacteria and was for a time investigated for use as an antibiotic (Cederlund and Mardh 1993; Elvers and Wright 1995). However, once again these inhibitory effects were found only at concentrations far above those found in the environment. Only in recent years has the toxicology of environmentally relevant concentrations of ibuprofen been investigated (Cleuvers 2004; Pomati, Netting et al. 2004; Richards, Wilson et al. 2004; Escher, Bramaz et al. 2005; Pomati, Castiglioni et al. 2006; Flippin, Hugget et al. 2007)

Ecotoxicological data indicate that the potential for acute or sub-acute toxicity is far too low to be of environmental relevance in a variety of species studied (Cleuvers 2004; Escher, Bramaz et al. 2005). However, investigations into subtle and mixture toxicity have suggested that some effects can be caused by environmentally relevant concentrations (Pomati, Netting et al. 2004; Richards, Wilson et al. 2004; Flippin, Hugget et al. 2007; Han, Choi et al. 2010)

A number of studies have found various effects on fish at concentrations approaching environmental relevance. For example, Flippen *et al.* (Flippin, Hugget et al. 2007) found that environmentally relevant ibuprofen concentrations caused subtle alterations in reproductive function in female Japanese medaka fish including increased clutch size and decresed egg development time, hypothetically due to ibuprofen's effects on cyclooxygenases and prostaglandin synthesis in vertebrate reproduction. Han *et al.* found that ibuprofen delayed hatching in Japanese medaka at concentrations as low as 0.1 µg/L (Han, Choi et al. 2010). David *et al.* (David and Pancharatna 2009) found that ibuprofen elicited a wide variety of developmental and behavioral effects in Zebrafish at 10ug/L.

Schnell *et al.* (Schnell, Bols et al. 2009) found that while they did not detect effects on trout cells at relevant concentrations of ibuprofen on its own, pharmaceutical mixture results suggested synergistic effects when ibuprofen was used in combination with other classes of drugs.

One of the most profound effects was described by Pomati *et al.* (Pomati, Netting et al. 2004) who found that ibuprofen strongly stimulated the growth of the cyanobacter *Synecohcystis,* causing a 72% increase in growth rate at an ibuprofen concentration of 10

ppb. The same study revealed a 25% growth reduction in the growth of the aquatic plant Duckweed (*Lemna minor*) at concentrations of ibuprofen that might be found in sewage treatment plant effluent. Results from both of these species suggest that primary producers, which are of high importance in oligotrophic aquatic ecosystems, may be sensitive to the effects of ibuprofen.

Richards *et al.* (Richards, Wilson et al. 2004) investigated the effects on aquatic community structure caused by mixtures of pharmaceuticals at environmentally relevant concentrations. The mixture that they tested included ibuprofen, fluoxitine (commonly known as prozac), and ciprofloxacin. The complexity and burdensomeness of doing mesocosm studies prohibited a large sample number and thus reduced statistical certainty. However, a mixture of the three compounds including ibuprofen at a concentration of 60 parts per billion caused significant effects on the aquatic community structure. There was an overall reduction in microbial diversity and algae became more predominant (Richards, Wilson et al. 2004).

Pomati *et al.* (Pomati, Castiglioni et al. 2006) investigated the effects of a complex mixture of thirteen pharmaceuticals, including ibuprofen, on human embryonic cells. The exposure concentrations were based on existing data regarding the highest reported riverine concentrations of the pharmaceuticals. A 10%-30% decrease in cell proliferation was observed with an acculation of cells at the G2/M phase and associated overexpression of cell cycling proteins *p16* and *p21*, indicating a disruption of cell cycling systems. The cells exhibited increased activation of stress response kinases and glutathione-S-transferase. While the exposure conditions were two steps removed from environmental relevance (maximum observed toxicant concentrations and raw exposure

of embryonic cells in the absence of an organismal context), the results indicate the potential for pharmaceutical mixtures to have subtle effects over short exposure times.

Other than the Pomati *et al.* (Pomati, Castiglioni et al. 2006) human embryonic cell study, the studies described above made no attempt to discover the mechanism by which ibuprofen was exerting effects on the target organisms. A number of studies have made the attempt to identify modes of ibuprofen toxicity using traditional toxicological methods aimed at identifying major toxic effects including mutagenesis, GSH induction, baseline toxicity (membrane disruption), and estrogenicity, but have only detected toxicity at concentrations far above those relevant in the environment (Cleuvers 2004; Escher, Bramaz et al. 2005). Therefore, the mechanism by which ibuprofen may have exerted effects upon the aquatic organisms at environmentally relevant concentrations remains completely unknown (Pomati, Netting et al. 2004; Richards, Wilson et al. 2004).

The organism which is the subject of this dissertation, *Sphinogmonas* Ibu-2, was enriched and isolated on media containing a much higher concentration (500 mg/L) of ibuprofen than is found in the environment. The low environmental concentration of pharmaceuticals may make the maintenance and expression of specific metabolic genes unfavorable in a selective sense, although it is not clear what the "cut-off" exposure level is for these compounds. Although this subject is beyond the scope of this dissertation, it is likely to be very important in determining whether and how pharmaceuticals are biologically transformed in the environment. That said, similar enrichment based studies have resulted in the isolation of organisms whose degradation pathway biochemistry has been shown to be relevant to pollutant degradation at environmentally relevant pollutant concentrations (Pumphrey and Madsen 2007). Thus, while more work is needed in order

to understand if the Ibu-2 metabolic paradigm presented below is relevant to more real world conditions, the research chapters presented here represent the first published effort to describe the biochemistry and genetics of ibuprofen degradation. It serves two general purposes; as an important first step in identifying metabolites to search for under more environmentally relevant conditions and as an investigation into what metabolic strategies might be employed by bacteria in the degradation of structurally related chemicals.

Bacterial metabolism of aromatic chemicals

Biosynthesis of the six-carbon aromatic ring is essential for the production of certain amino acids and diverse biological cofactors, though many aromatic compounds in the environment are of pyrogenic or petrogenic origin. A large percentage of lignin, which is the second-most common biological compound on the surface of the planet (Kirk and Farrell 1987) is also aromatic, being primarily composted of phenolic heteropolymers (Masai, Katayama et al. 2007). "Aromatic" refers specifically to circular hydrocarbons and heterocycles with delocalized π-orbital electrons (Vaillancourt, Bolin et al. 2006; Phale, Basu et al. 2007); while these are reduced, energy-rich compounds, they are unusually resistant to chemical attack due to the negative resonance of the delocalized electrons and the inaccessibility of the carbons (Vaillancourt, Bolin et al. 2006; Phale, Basu et al. 2007). Presumably because they are energy rich and ubiquitous, bacterial pathways for metabolizing aromatic compounds are common. However, the

unusual stability of the ring necessitates highly specialized enzymatic machinery for catalysis.

The bulk of the scientific work examining the microbial metabolism of aromatic chemicals has tended to point to certain rules or trends common to the metabolic strategies that bacteria employ. A simple, if inaccurate, model for aerobic bacterial degradation of the aromatic group is that of the double-dioxygenation; an initial addition of two oxygens to the aromatic group seems sufficient to destabilize the structure and allow for a second dioxygenation event to cleave the ring, which prepares the resulting non-aromatic metabolite for catabolism and eventual entry into central carbon cycling pathways (Masai, Katayama et al. 2007; Phale, Basu et al. 2007; Fuchs 2008; Zeyaullah, Abdelkafe et al. 2009; Ju and Parales 2010). Presumably the initial double oxygenation, whether it be a single hydroxylation of an already-oxidized ring, sequential monoxygenations, or a single dioxygenation event, is required to destabilize the ring, perhaps by altering the resonance of the delocalized π-orbital electrons or by deshielding the ring-carbons (Parales and Resnick 2006; Phale, Basu et al. 2007). In actuality, the double-dioxygenation paradigm is not by any means universally applicable, though this paradigm holds true for the aerobic degradation of simple aromatic chemicals such as benzene and toluene and remains a likely first hypothesis for predicting the mechanism of an unknown degradative pathway.

Classical double-dioxygenation metabolism of aromatics

While dihydroxylation of the aromatic ring itself is a nearly universal prerequisite for ring cleavage , microbes possess a diverse array of mechanisms for doing this

(Yamaguchi and Fujisawa 1978; Schweizer, Markus et al. 1987; Butler and Mason 1997) (Figure 1.4). The two hydroxyl groups can be added in adjacent or opposite positions upon the ring. In the case of aromatic chemicals that do not already have hydroxyl groups, simultaneous dioxygenation via multicomponent dioxygenase and subsequent dehydrogenative rearomatization is the typical route of transformation (Butler and Mason 1997; Parales and Resnick 2006). Aromatic compounds that have two adjacent hydroxyl groups are commonly termed catechols. There are fewer examples of opposite position, or *para*-, hydroxylations such as the homogentisate (2,5-dihydroxyphenylacetic acid) pathway that is commonly used by both eukaryotes and prokaryotes in aromatic amino acid metabolism (Arias-Barrau, Olivera et al. 2004) and in the metabolism of alkylphenols via hydroquinone by an environmental isolate *Sphingomonas* sp. strain TTNP3 (Corvini 2006).

The initial dioxygenation is a reductive hydroxylation requiring high-energy electron input (Parales and Resnick 2006; Kweon, Kim et al. 2008). This power is typically supplied by ferredoxins which are in turn powered by NAD(P)H dependent ferredoxin (FeDox) reductases (Mason and Cammack 1992; Kweon, Kim et al. 2008). The typical electron transfer chain is NAD(P)H > FeDox-Reductase > Ferredoxin > O^2 + substrate. Ferredoxins and their reductases are both redox-active iron-sulfur proteins. Most reductases studied to date use NADH, a ubiquitous reduced nucleotide-based cofactor, as an electron source. While an electron source is required for the initial dioxygenation event, NADH is subsequently regenerated in the following dehydrogenation event (Figure 1.4) (Parales and Resnick 2006), making the conversion of the aromatic to a catechol net energy-neutral.

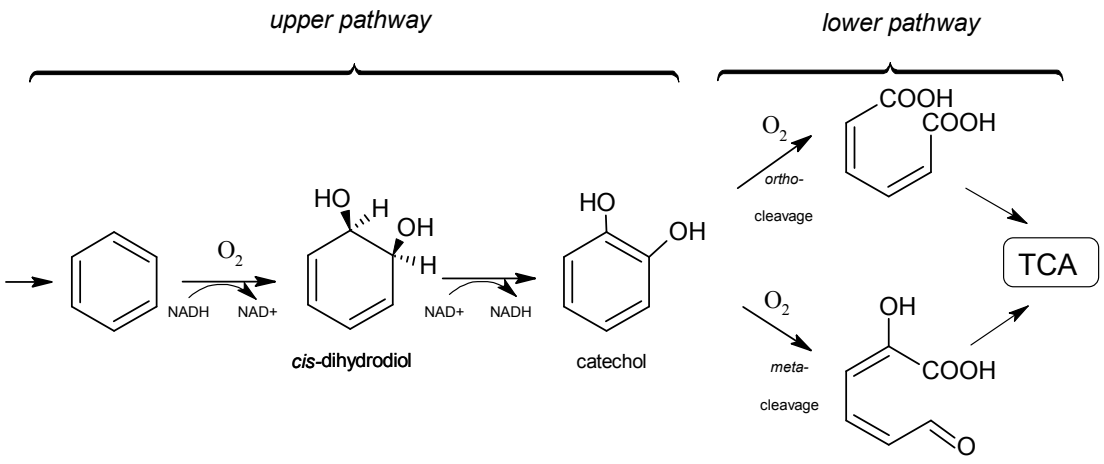

Figure 1.4. Basic features of the double-dioxygenation paradigm for the aerobic metabolism of aromatic chemicals by bacteria. "Upper pathway" broadly refers to the reactions leading up to initial ring dioxygenation while "lower pathway" refers to all reactions following the initial dioxygenation.

Catechols are the substrates for further metabolism by other types of dioxygenases (Figure 1.4). These dioxygenases do not require an electron transport chain, presumably because the destabilized ring is vulnerable to attack at this stage. Ring cleavage in the 2,3 position relative to the two hydroxyl groups of a catechol is termed *meta*- or extradiol- cleavage, to indicate that fission takes place adjacent to the two hydroxy groups. The ring fission product of a meta-cleavage reaction exhibits a diagnostic yellow color that disappears upon acidification. The alternative to meta-cleavage is *ortho*- or intradiol- cleavage, in which the ring is broken between the two hydroxy groups. In this situation, no yellow product is observed (See (Harayama, Kok et al. 1992) and (Vaillancourt, Bolin et al. 2006) for reviews of ring-cleavage dioxygenases).

The most-studied aromatic acid degradation processes so far involve an initial dioxygenation in the 1,2 or 2,3 position. An example of 1,2 dioxygenation is found in the

TOL pathway of *Pseudomonas putida* mt-2. The TOL operon encodes for the genes for the degradation of toluene and the xylenes. Toluene is sequentially oxidized at the methyl group to benzoate. Benzoate is then *cis*-dioxygenated in the 1,2 position to cis-benzoate dihydrodiol, which is decarboxylated and dehydrogenated to form catechol (1,2-dihydroxybenzene). Catechol is then dioxygenated at the 2,3 position, cleaving the ring (Eaton 1996; Eaton 1997).

An example of a 2,3-dioxygenation pathway for the degradation of an aromatic acid is the degradation of 4-isopropylbenzoate (cumate). It is the only aromatic acid with a branched aliphatic substituent in the *para*-positionwhose degradation pathway has been fully described. Cumate is dioxygenated at the 2,3 position by *Pseudomonas putida* F1 via enzymes encoded by the *cmt* operon. It is then subsequently dehydrogenated to yield 2,3-dihydroxy-4-isopropylbenzoate (Figure 1.5). This compound is then cleaved via dioxygenation across the 3,4-bond, a *meta*-cleavage that yields a diagnostic pH-dependent yellow product (Defrank and Ribbons 1976; Defrank and Ribbons 1977; Eaton 1996; Eaton 1997).

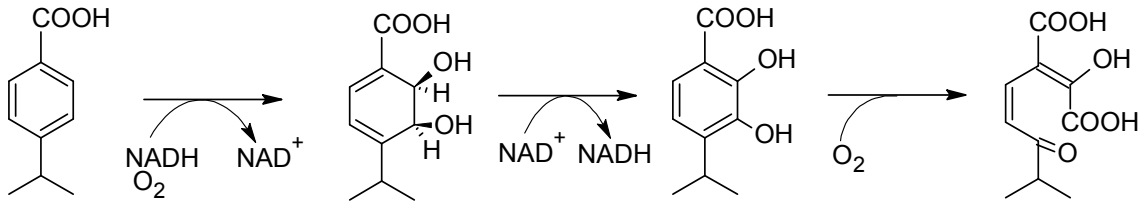

Figure 1.5. General scheme of the 1,2 dioxygenation *cmt* pathway (Eaton 1996; Eaton 1997).

Alternatively there are a number of aromatic metabolic strategies that seem to not necessarily require the initial double-oxygenation or even that the ring be oxygenated at

all. A large portion of biological chemistry, from both a current spatial perspective and certainly in regards to the geological time-frame, occurs under anoxic conditions. Even though oxygen is the most potent electron acceptor on the modern earth, nitrate and ferric iron are both useful and reasonably abundant terminal electron acceptors with sufficient oxidizing power to permit their use during the break down aromatic chemicals by bacteria (see.(Foght 2008; Fuchs 2008; Carmona, Zamarro et al. 2009) for current reviews). Clearly in the absence of ubiquitous oxygen gas, the double-dioxygenation paradigm becomes irrelevant, leading to necessarily different strategies for ring-opening.

Alternative aerobic ring cleavage substrates

Some facultative anaerobes actually utilize the same metabolic system under both aerobic and anaerobic conditions. Bacteria couple a benzoate CoA-ligase mediated first step to a variation of the double-dioxygenation paradigm in which an oxygenase adds dioxgyen across the 2,3 bond while simultaneously dearomatizing the ring, followed by a direct, non oxygen-dependent ring cleavage (Gescher, Eisenreich et al. 2005), while the aerobic metabolism of phenylacetic acid further blurs the line between the two paradigms as described below.

There are also other examples of aerobic aromatic ring-openings, namely those of salicylate (Hintner, Lechner et al. 2001) and aminoaromatics (Kulkarni and Chaudhari 2007; Ju and Parales 2010) that do not require an intial ring dioxygenation. Additionally, there are a number of examples of pathways that actually utilize trihydroxybenzenes as ring cleavage substrates that, while they do not as dramatically challenge the dihydroxylation model, are variations that should be acknowledged (Zeyer and Kearney

1984; Kluge, Tschech et al. 1990; Hanne, Kirk et al. 1993; Stolz and Knackmuss 1993; Meulenberg, Pepi et al. 1996; Kadiyala and Spain 1998; Haigler 1999; Johnson, Jain et al. 2000; Schink, Phillips et al. 2000; Dayan, Watson et al. 2007; Kulkarni and Chaudhari 2007; Carmona, Zamarro et al. 2009).

Anaerobic metabolism of aromatics

As in aerobic aromatic degradative strategies, the anaerobic reactions responsible for aromatic ring cleavage can also be grouped into upper pathways that converge upon a central ring cleavage substrate and a lower pathway that is responsible for ring cleavage and further degradation (Foght 2008; Fuchs 2008; Carmona, Zamarro et al. 2009). While for a variety of reasons anaerobic pathways have not been as extensively studied as aerobic pathways, the existing evidence seems to suggest that a large number of unique strategies are employed in upper pathway metabolism (Carmona, Zamarro et al. 2009). This may be rationalized in part as the result of the fact that the term "anaerobic" is a catch-all term for pathways that utilize a very wide variety of electron acceptors with very different redox potentials and thus different energetic conditions. For example, upper pathways adapted for using nitrate or ferric iron as electron acceptors (Bouwer and Zehnder 1993; Wilson and Bouwer 1997; Chakraborty and Coates 2004), both of which have moderately high redox potentials, are quite different from those adapted to less ideal electron acceptors such as sulfate (Diaz 2000).

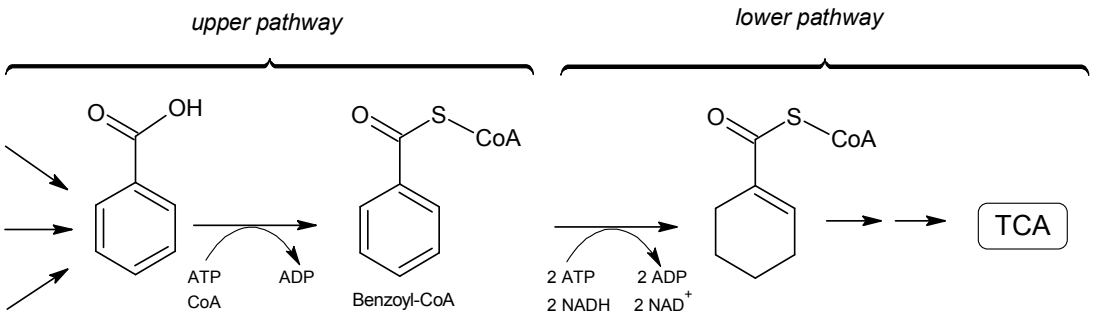

Figure 1.6. General scheme of the benzoyl-CoA paradigm for the anaerobic metabolism of aromatic chemicals by bacteria (Carmona, Zamarro et al. 2009).

Through a wide variety of strategies, anaerobic aromatic metabolism is funneled through a single central metabolite regardless of redox conditions; benzoyl-coenzyme-A (Figure 1.6) (Carmona, Zamarro et al. 2009). Though there is scant evidence so far, it appears that even benzene is converted to benzoyl-CoA under anaerobic conditions, so universal is this metabolic intermediate (Ulrich, Beller et al. 2005). Benzoyl-CoA is cleaved via a drastically different mechanism than that observed in classical aerobic examples. Benzoyl-CoA is cleaved by an energy-dependent reductive hydrolytic mechanism that requires reduced ferredoxin and ATP (reviewed by Boll (Boll 2005).) The ATP-dependent CoA-ligation (or non-energy-dependent CoA-transfer in some cases (Muller and Schink 2000; Leutwein and Heider 2001)) is believed to activate the ring for reductive hydrolysis in a manner analogous to the oxidative activation of the ring for oxidative ring cleavage in addition to facilitating downstream metabolism (Vellemur 1995). CoA-ligation may also drive uptake and retention by keeping the intracellular concentration of the substrate effectively low, a strategy generally termed vectoral acylation (Black and DiRusso 2003; Zou, F. et al. 2003)

Metabolism of phenylacetate

Recently-described pathways for the bacterial catabolism of phenylacetic acid have also been shown to utilize hydrolytic ring cleavage mechanisms similar to those found in anaerobic pathways (Teufel, Mascaraque et al. 2010). Phenylacetic acid is a relatively simple chemical which upon initial inspection does not seem to warrant catabolic strategies vastly different from those required for the other simple aromatics such as BTEX (benzene, toluene, ethylbenzene, xylene) or benzoates. Indeed, early investigations into phenylacetate biodegradation yielded circumstantial evidence for the utilization of pathways involving homoprotocatechuate (3,4-dihydroxyphenylacetic acid) or homogentisate (2,5-phenylacetic acid) (Roof, Lannon et al. 1953; Kunita 1955; Kunita 1955)

Ibuprofen is similar in nature to phenylacetic acid in that the acid group is two carbons removed from the aromatic ring. As described below, it was believed until fairly recently that bacteria metabolize phenylacetic acid through the same pathways by which they metabolize hydroxyphenylacetic acids, which they convert via monooxygenation to either 3,4-hydroxyphenylacetic acid (homoprotocatechuate) or 2,5-hydroxyphenylacetic acid (homogentisate) (Sparnins, Chapman et al. 1974; Sparnins and Chapman 1976; Wegst, Tittman et al. 1981; Arias-Barrau, Olivera et al. 2004). Homoprotocatechuate and homogentisate intermediates were first identified during early investigations into the biochemistry of fungal (Kluyver and van Zijp 1951; Perrin and Towers 1973), animal (Knox and Edwards 1955; Crandall, Krueger et al. 1960; Flamm and Crandall 1963; McCormick, Young et al. 1965), and bacterial (Roof, Lannon et al. 1953; Kunita 1955; Kunita 1955; Jamaluddin 1977; Lee Y-L and Dagley 1977) metabolism.

Fungal metabolism of phenylacetate

The capacity of fungi to perform assimilative metabolism of phenylacetic acid via homogentisate is well-established. In 1976, Kishore *et al.* (Kishore, Sugumaran et al. 1976) demonstrated that the fungus *Aspergillus niger* converted phenylalanine to phenylacetic acid, 2- and 4-hydroxyphenylacetic acid, and homogentisate by performing thin layer chromatography on radiolabelled substrate. They additionally demonstrated that both phenylacetic acid hydroxylase and homogentisate oxygenase activites were upregulated in phenylalanine-grown cultures, offering strong support to the assertion that phenylacetate can be metabolized via homogentisate in fungi. This model was further supported by the subsequent characterization of phenylacetate 2-hydroxylase mixed function NADPH-dependent p450 enzyme in phenylacetic acid grown *A. niger* cultures (Sugumaran and Vaidyanathan 1979) and in other *Aspergillus* species (Fernandez-Canon and Penalva 1995; Mingot, Penalva et al. 1999; Rodriguez-Saiz, Barredo et al. 2001). The homogentisate ring-cleavage dioxygenase has also been isolated from fungi (Fernandez-Canon and Penalva 1995). A similar pathway involving the sequential mono-oxidation of ethylbenzene to homogentisate via phenylacetic acid has also been described in the fungus *Exophiala lecanii-corni* (Gunsch, Cheng et al. 2003; Gunsch, Cheng et al. 2005).

Early research into the bacterial metabolism of phenylacetate

In two of the earliest reports, Kunita (Kunita 1955; Kunita 1955) reported that *Pseudomonads* had the capacity to metabolize phenylacetate via both homogentisate and protocatechuate pathways, though later it was reported that *Pseudomonas* might

alternatively metabolize phenylacetic acid via a decarboxylative mechanism to catechol via benzoate based on detection of putative metabolites in the media (Sariaslani, Sudmeier et al. 1982). In the 1950's, Dagley *et al.* reported that *Vibrio* also metabolized phenylacetic acid via homogentisate, though the conclusion was based solely upon an apparently high level of oxygen consumption in washed phenylacetate-grown cells exposed to homogentisate (Dagley, Fewster et al. 1953). However, such up-regulation was measured in the absence of any negative controls making the conclusion tenuous. The authors never actually demonstrated the conversion of phenylacetate to homogentisate (Chapman and Dagley 1962). Later, Boer *et al.* found evidence in support of this general model in *Nocardia*, though using the same single experimental technique; oxygen consumption of induced washed-cell systems exposed to homogentisate (Boer, Harder et al. 1988). Pometto *et al.* (Pometto and Crawford 1985) did offer stronger support for the employment of the homogentisate model in *Streptomyces* sp. by first demonstrating that phenylacetate accumulated in phenylalanine-grown cultures, leading to the conclusion that phenylacetate is an intermediate of phenylalanine in such bacteria. They further demonstrated that homogentisate dioxygenase activity was higher in phenylalanine grown cells than in tyrosine grown cells. Once again however, there were no negative controls utilized and no statistical testing was employed to support the claims, nor was any direct conversion of phenylacetate to homogentisate achieved (Pometto and Crawford 1985). A 1988 study by van den Tweel *et al.* (van den Tweel, Smits et al. 1988) again claimed to find evidence for aerobic metabolism of phenylacetate via homogentisate based on up-regulation of associated oxygenase activities in the absence of any direct phenylacetate hydroxylase activity by induced cell-free extracts.

In 1985, Cooper *et al.* suggested that the then current model of phenylacetate metabolism via homogentisate or protocatechuate was not tenable because associated dioxygenase activities by phenylacetate grown *Pseudomonads* were negligible. The same group had definitively demonstrated that 3- and 4-hydroxyphenylacetate were metabolized by *E. coli* via protocatecuate (Cooper and Skinner 1980). However in the case of phenylacetate-grown *E. coli* they reported an accumulation of 2-hydroxyphenylacetate, which was a dead-end metabolite, and no enzymatic activities or metabolites indicative of either the protocatechuate or homogentisate pathways being utilized (Cooper, Jones et al. 1985). As described below, it would later be repeatedly shown that this enigmatic 2-hydroxy dead-end metabolite accumulates in several phenylacetate-degrading bacteria and is dead-end side product of the true underlying metabolic pathway.

Phenylacetyl coenzyme A ligase pathway

In the early 1990s several studies published out of Dr. Georg Fuchs's laboratory characterized phenylacetyl-coenzyme A ligases utilized under anaerobic conditions by *Pseudomonas* (Dangel, Brackmann et al. 1991; Seyfried, Tschech et al. 1991; Mohamed and Fuchs 1993; Mohamed, Seyfried et al. 1993). Induction of phenylacetyl-coenzyme A ligases under anaerobic conditions were also reported in *E. coli* (Vitovski 1993), *Acinetrobacter* (Vitovski 1993), *Alcaligenes* (Vitovski 1993), and later, *Rhodococcus* (Navarro-Llorens 2005), *Silicibaacter* (Yan, Kang et al. 2009), and *Thermus thermophilus* (Erb, Ismail et al. 2008). As described above, coenzyme A (CoA) is a nucleotide-based cofactor utilized in a wide variety of metabolic systems throughout all

branches of life (Vellemur 1995; Leonardi, Zhang et al. 2005; Spry, Kirk et al. 2008). CoA is utilized by bacteria under anaerobic conditions to activate aromatic substances for ring-cleavage and subsequent catabolism and possibly also to drive uptake (Vellemur 1995; Carmona, Zamarro et al. 2009). Dangel *et al.* (Dangel, Brackmann et al. 1991) demonstrated using radiolabelled substrate that *Pseudomomas* activates phenylacetic acid under anaerobic conditions using a coenzyme A ligase and then proceeds to decarboxylate it to benzoyl-coenzyme A with equimolar release of radiolabelled CO_2 gas via a phenylglyoxylate intermediate (Figure 1.7), whereas Seyfried *et al.* (Seyfried, Tschech et al. 1991) showed that several environmental *Pseudomonas* isolates obtained from an anaerobic phenylacetate enrichment were able to metabolize a variety of putative intermediate compounds that were consistent with the decarboxylative benzoyl-coenzyme A pathway.

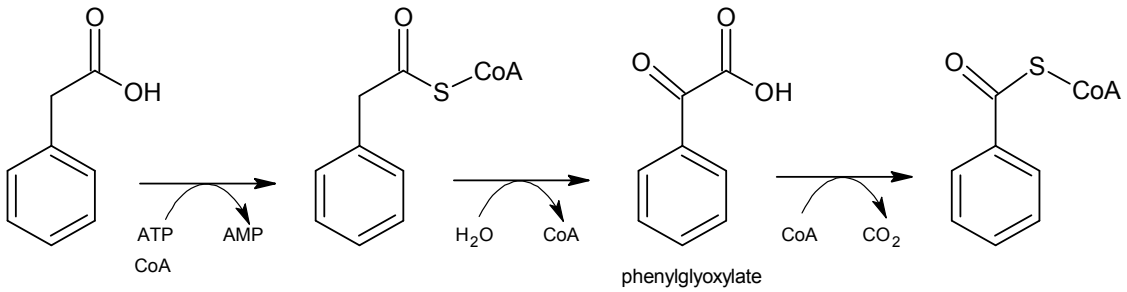

Figure 1.7. The phenylglyoxylate pathway (Dangel, Brackmann et al. 1991), which channels phenylacetate to the central benzoyl-CoA pathway under anaerobic conditions in *Pseudomonas* (Dangel, Brackmann et al. 1991), *Thauera aromatica* via (Hirsch, Schagger et al. 1998) and *Azoarcus evansii* (Hirsch, Schagger et al. 1998)

In the same *Pseudomonas* system in the same laboratory, Mohamed *et al.* (Mohamed and Fuchs 1993) characterized phenylacetyl-CoA ligase activity using chromatographic methods, showing considerable up-regulation of the activity in induced cells versus glucose-grown cells. Curiously, the group reported that the activity was also induced

under aerobic conditions to a lesser extent, but discarded the information as artifactual. In the following years, several more projects came out of the Fuchs lab describing in detail the biochemistry of metabolism of phenylacetyl-coenzme A to benzoyl-coenzyme A via phenylglyoxylate under anaerobic conditions in *Thauera aromatica* (Hirsch, Schagger et al. 1998) and *Azoarcus evansii* (Hirsch, Schagger et al. 1998). It would shortly be reported that aerobically-grown *Azoarcus evansii* also employs a phenylacetyl-CoA ligase (Mohamed 2000; Rost, Haas et al. 2002) by demonstrating the *in vivo* CoA-ligase activity of radiolabelled compounds preferentially by phenylacetate-induced cells (Mohamed 2000).

A short time previously, a Spanish research group led by Dr. Jose Luengo described the induction of a phenylacetyl-coenzyme A enzyme in aerobically-grown *Pseudomonas putida* U (Martinez-Blanco, Reglero et al. 1990). The group concentrated the enzymatic fraction of induced cell cultures and demonstrated ATP and Mg^{2+} dependent conversion of phenylacetate to phenylacetyl-coenzyme A via HPLC. At the time, such an intermediate in an aerobic aromatic pathway was both unprecedented and ran counter to the accepted model of metabolism via homogentisate or protocatechuate; accordingly, the researchers hedged by stating that such enzymatic activity might actually be due to a non-specific activity or a detoxification mechanism (Martinez-Blanco, Reglero et al. 1990). This uncertainty was shortly cleared up with the demonstration that genetic knockout mutants that had lost the ability to grow on phenylacetate accordingly lost the ability to generate phenylacetyl-coenzyme A (Schleissner, Olivera et al. 1994). The 1.3 kilobase phenylacetyl-coenzyme A ligase gene with high similarity to acetyl-CoA ligases and fatty acid-CoA ligases was identified in *Pseudomonas putida* U via Tn5

transposon mutagenesis (Minambres, Martinez-Blanco et al. 1996). A nearly identical gene (86% identity) was identified in *P. putida* Y2 wherein it is necessary for the metabolism of styrene via phenylacetate (Velasco, Alonso et al. 1998).

Shortly thereafter, several genes associated with aerobic phenylacetate metabolism in *P. putida* U (Olivera, Minambres et al. 1998) and the styrene-metabolizer *P. putida* Y2 (Alonso, Bartolomé-Martín et al. 2003; Bartolomé-Martín, Martínez-García et al. 2004) were identified and sequenced. In addition to the coenzyme A ligase that had already been described (*phaE*), four genes associated with ring hydroxylation (*phaFGHI*) and a gene encoding a putative ring-opening enzyme (*phaL*) were identified. The putative ring hydroxylation genes were identified based on conserved oxidase and electron transfer domains and by the fact that when they were knocked out, phenylacetate accumulated during the metabolism of chemicals degraded via a phenyacetyl-coenzyme A intermediate (Olivera, Minambres et al. 1998). When *phaL* was knocked out, 2-hydroxyphenylacetate accumulated in the media, leading to the tentative conclusion that this monohydroxylated chemical was actually an intermediate (later shown to be wrong as described below) upon which the *phaL* gene product performed a ring fission though sequence analysis revealed no similarity to any known ring-fission associated genes (Olivera, Minambres et al. 1998).

Genes closely related to those of the Pha operon in *P. putida* U were also discovered in the aerobic phenylacetate-metabolizer *E. coli* W (Ferrandez, Minambres et al. 1998; Olivera, Minambres et al. 1998); a phenylacetyl-coenzyme A ligase (*paaK*), a multicomponent oxygenase (*paaABCDE*), and putative lower-pathway-encoding genes (*paaGJZ*). Sequence analysis of the putative *paaB* and *paaE* gene products showed

rieske iron clusters and conserved FAD+ and NAD(P)+ binding domains with similarities to the electron transport components of some toluene and phenol mono-oxygenases, suggesting that these two genes encode an electron transport system. *paaACD* showed no similarities to any functionally characterized genes and were hypothesized to encode a multicomponent phenylacetyl-coenzyme A oxygenase. Knocking out *paaG, paaJ,* or *paaZ* resulted in accumulation of 2-hydroxyphenylacetate which was clearly a dead-end metabolite in *E. coli* W. *paaG* and *paaJ* showed similarities to genes that encode beta-oxidation enzymes, enoyl-CoA hydratase/isomerase and beta-ketoadipyl-CoA thioloase respectively and as such were expected to be involved in metabolizing the product of ring cleavage. By default, PaaZ appeared to be the ring-opening enzyme, though it bore no resemblance to any known ring-opening enzyme; rather, like *phaL* (Olivera, Minambres et al. 1998), it bore only a conserved aldehyde dehydrogenase domain, and ultimately provided no clear hypothesis regarding how ring-opening might occur (Ferrandez, Minambres et al. 1998). Mohammed *et al.* would later show that similar genes and metabolites were present in *Azoarcus evansii, Escherichia coli, Rhodopseudomonas palustris* and *Bacillus stearothermophilus,* indicating that this coenzyme A mediated pathway is widely distributed in bacteria (Mohamed, Ismail et al. 2002). Again the accumulation of the apparent dead-end metabolite 2-hydroxyphenylacetate was observed, though it was postulated that it might be an abiotic breakdown product of 2-hydroxyphenylacetyl-CoA which might be a true metabolic intermediate (Mohamed, Ismail et al. 2002).

Rost *et al.* attempted to identify the ring-cleavage substrate by knocking out *pacL*, the putative gene thought to encode phenylacetate ring-cleavage activity in *A. evansii* (the

equivalent of *paaZ* in *E. coli* W and *phaL* in *P. putida* U) (Rost, Haas et al. 2002). In the knockout mutant, a compound identified via NMR as 2,4,6-cycloheptatriene-1-one was identified, though no attempts to see whether or not this was metabolizable by induced cells was reported. The authors suggested that this unexpected accumulating metabolite was the product of rearrangement of the actual ring-cleavage substrate by housekeeping enzymes or abiotic reaction (Rost, Haas et al. 2002). Ismail *et al.* attempted to solve the ring-opening question using the same tactics (Ismail, Mohamed et al. 2003); NMR analysis of knockout mutants in *E. coli* K12. As had been previously reported (Olivera, Minambres et al. 1998; Mohamed, Ismail et al. 2002), *paaG* and *paaZ* knockouts accumulated 2-hydroxyphenylacetate. However, an additional metabolite was detected in *paaG* knockouts; the gamma-lactone of 2-hydroxyphenylacetate (Figure 1.8 compound 4). Although PaaG was expected to act upon the ring-opened metabolite due to its similarity to beta-oxidation enoyl-CoA hydratase/isomerase proteins, the accumulation of a unique eight-carbon aromatic compound (Figure 1.8 structure 2) suggested instead that it might actually catalyze ring-opening via an isomerization of an activated aromatic-CoA compound. The structure of the lactone was consistent with the abiotic dehydration of a dearomatized 1,2-dihyroxy metabolite as is classically utilized in aerobic aromatic pathways by bacteria (Gibson 1968), though the postulated 1,2-dihydroxy was not detected directly (Ismail, Mohamed et al. 2003). The 2,4,6-cycloheptene-1-one metabolite detected by Rost *et al.* (Rost, Haas et al. 2002) was also postulated to be an abiotic breakdown product of the putative 1,2-diol intermediate (Ismail, Mohamed et al. 2003).

Further analysis of the putative multicomponent oxygenase system *paaABCDE* from *E. coli* (Fernandez, Ferrandez et al. 2006) expanded upon and supported the putative role that these genes play in phenylacetate metabolism. Sequence and phylogenetic analyses placed this five-gene operon firmly within the bacterial diiron multicomponent oxygnase (BMO) family, which contains such multicomponent oxygenases that monooxygenate methane, ammonia, linear alkanes, tetrahydrofuran, phenol, and toluene. While it had been postulated that this phenylacetyl-CoA oxygenase performed a dioxygenation, the sequence analyses strongly suggested a monooxygenation mechanism (Fernandez, Ferrandez et al. 2006). Indeed, a 2010 report by the Fuchs lab offered strong support for a monooxygenation mechanism of a sort unprecedented in aromatic metabolism (Teufel, Mascaraque et al. 2010). When Teufel *et al.* exogenously expressed the *paaABCDE* operon from *E. coli* K12 and exposed purified enzymatic reaction to radiolabelled phenylacetyl-CoA, an epoxide, 1,2-epoxyphenylacetyl-CoA (Figure 1.8 compound 11) was identified via NMR analysis. Enzymatic assay with labelled oxygen gas or labelled water revealed that the source of the epoxy-oxygen was diooxygen rather than water. Sequence analysis supported the previous predictions (Olivera, Minambres et al. 1998; Fernandez, Ferrandez et al. 2006) that PaaACD encodes for a multicomponent BMO while PaaB and PaaE play the electron-transfer role. The facts that the 2-hydroxyphenylacetic acid and lactone metabolites previously observed in ring-cleavage deficient mutants (Rost, Haas et al. 2002; Ismail, Mohamed et al. 2003) were also consistent with abiotic degradation of this epoxy metabolite and that the epoxide was more consistent with the conserved monooxygenase character of the ring cleavage operon *paaABCDE* (Fernandez, Ferrandez et al. 2006) appeared to have resolved the question of

how ring hydroxylation proceeds and is summarized in Figure 1.8.

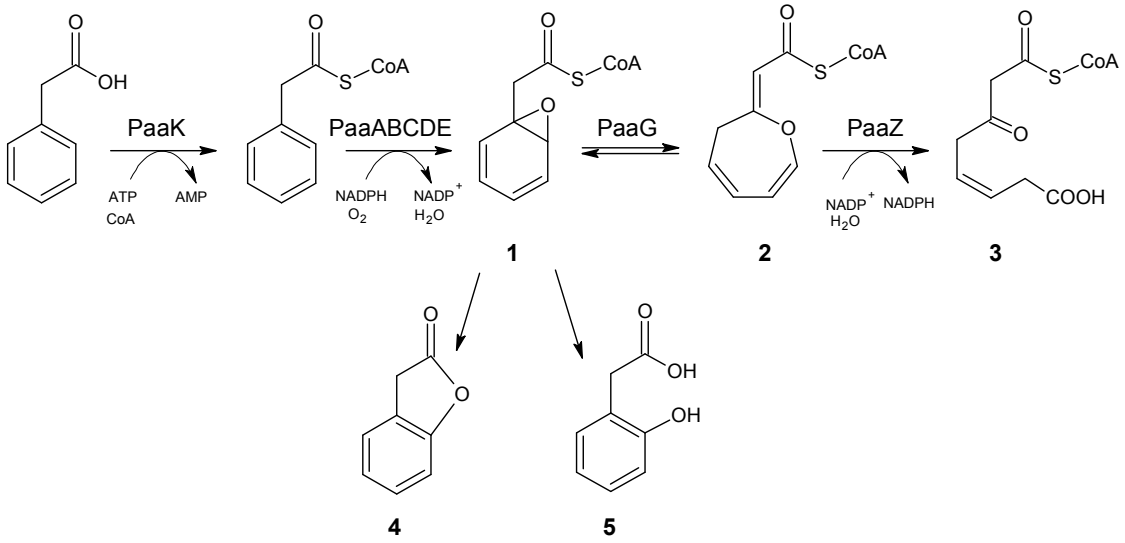

Figure 1.8. The *paa* pathway for the aerobic metabolism of phenylacetate (Teufel, Mascaraque et al. 2010).

The NMR experiments upon *E. coli* K12 *paa* clones and mutants by Teufel *et al.* (Teufel, Mascaraque et al. 2010) also cast light upon the mechanism of ring opening catalyzed by PaaG and PaaZ. In a manner somewhat consistent with it's conserved enoyl-CoA hydratase/isomerase identity, purified PaaG catalyzed the isomerization of the epoxyphenylacetyl-CoA to 2-oxepin-2-(3H)-ylideneacetyl-CoA(oxepin-CoA) (Figure 1.8 compound 2), a reversible reaction. Further addition of purified PaaZ catalyzed the NADP+-dependent conversion of epoxide and oxepin-CoA into 3-oxo-5,6-dehydrosuberyl-CoA (Figure 1.8 compound 3). Previous studies had identified only an aldehyde dehyrdrogenase domain in PaaZ (Ferrandez, Minambres et al. 1998), though further sequence analysis by Teufel *et al.* (Teufel, Mascaraque et al. 2010) revealed an additional enoyl-CoA-hydratase (MaoC) domain which the authors took to indicate that final ring cleavage was accomplished in a multi-step reaction catalyzed by PaaZ in which

the MaoC hydratase domain adds water across the C2-C3 bond, the ring is cleaved by the tautomerization of the unstable reaction product, and then the aldehyde dehydrogenase domain futher oxidizes the resulting aldehyde at the C8 position in an NADP+ dependent manner to produce the observed final metabolite. While the multi-step proposed PaaZ mechanism will require much more experimentation to establish firmly, the formation of an epoxy-CoA by PaaABCDE, the oxepin by PaaG, and ultimate ring cleavage by PaaZ was clearly demonstrated (Teufel, Mascaraque et al. 2010).

The presence of *paa*-like genes in 16% of sequenced bacterial genomes suggests that this CoA-ligase hydrolytic ring-cleavage mechanism may be a central paradigm for the aerobic metabolism of aromatic chemicals (Teufel, Mascaraque et al. 2010). Aerobic coenzyme A mediated metabolism of benzoate derivatives has also been described as mentioned above (Fuchs 2008). Such pathways are currently regarded as "hybrid" because they combine aspects of both a classical anaerobic (CoA ligation and hydrolytic ring cleavage) with aspects of typical aerobic (oxygenation of the aromatic ring) pathways (Fuchs 2008). The PAA hybrid pathway was particularly difficult to characterize, likely due to the novelty of the enzymes involved and the ephemeral qualities of the epoxy and oxepin metabolites produced. The true extent of its relevance has therefore been clouded however based on the prevelance of uncharacterized *paa* homologs in the genomes of sequenced bacteria, it is becoming clear that similar hybrid paradigms for the metabolism of aromatic acids are wide spread and may be as common as the better characterized double-dioxygenation and anaerobic benzoyl-CoA paradigms.

Bacterial metabolism of 2-phenylpropionate

Even more structurally similar to ibuprofen than phenylacetate is 2-phenylpropionate. However, data regarding the microbial degradation of phenylpropionate is much scarcer than that regarding phenylacetate. Site-specific hydroxylation of phenylpropionate has been observed in the gram-positive bacterium *Streptomyces rimosus*, which converts phenylpropionate to 4-hydroxyphenylpropionate (Kuge, Mochida et al. 1991). *Pseudomonas cepacia* has been shown to metabolize phenylpropionate and tropic acid (2-phenyl-3-hydroxypropionic acid) via decarboxylation of the alpha methyl group to phenylacetaldehyde followed by oxidation to phenylacetate (Andreoni, Baggi et al. 1992). Long *et al.* (Long 1997) studied in detail the steps involved in the oxidation of tropic acid to phenylacetate by *Pseudomonas* sp. strain AT3. They demonstrated that tropic acid is dehydrogenated to phenylmalonic semialdehyde (2-phenyl-3-oxopropionic acid), decarboxylated to phenylacetaldehyde, and dehydrogenated to phenylacetic acid.

Metabolism of ibuprofen

Little information exists regarding how ibuprofen is oxidatively metabolized by environmental microbes. The fungus *Verticillum lecanii* has been shown to hydroxylate ibuprofen on the isobutyl group prior to metabolism, forming (S)-2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (Hanlon, Kooloobandi et al. 1994). In biofilm reactor experiments, Zweiner *et al.* (Zwiener, Seeger et al. 2002) and Winkler *et al.* (Winkler, Lawrence et al. 2001) reported a small increase in the concentration of hydroxyibuprofen and carboxyibuprofen concomitant with the decrease in concentration

of ibuprofen under aerobic conditions. Quintana *et al.* (Quintana, Weiss et al. 2005) also reported identifying two isomers of hydroxyibprofen in spiked active sewage sludge. Short-lived intermediates such as substituted catechols would have not been detected using the techniques described in any of the studies listed above, nor was the generation of the hydroxylated metabolites linked to assimilative metabolism. This data suggests that microbes could be metabolizing ibuprofen through a mono-oxygenation process similar to that observed in *Verticillum lecanii* (Hutt, Kooloobandi et al. 1993). However, in the Zweiner *et al.* study (Zwiener, Seeger et al. 2002) the hydroxy- and carboxyibuprofen accounted for only 7% of the ibuprofen that was degraded. How that remaining 90+% was metabolized was not determined.

Chapter two of this dissertation provides evidence detailing a unique metabolic pathway for the degradation of ibuprofen whereby the acid side chain is removed and a catecholic intermediate is formed. Chapter three details the genes found in *Sphinogmonas* Ibu-2 which encode the observed metabolic pathway described in chapter two.

Transport of aromatics and related chemicals by bacteria

Most aromatic acid transport systems studied to date have been found to utilize inner membrane porins of the major facilitator superfamily (MFS) that tend to couple transport to the influx of hydrogen ions (Marger and Saier 1993; Pao, Paulsen et al. 1998; Kahng, Byrne et al. 2000; Kasai, Inoue et al. 2001). Such proteins have been found to be required for the uptake of phthalate (Chang, Dennis et al. 2009), benzoate (Clark, Momany et al. 2002; Ledger, Flores-Aceituno et al. 2009; Wang, Xu et al. 2011),

chlorobenzoate (Ledger, Flores-Aceituno et al. 2009), 4-hydroxybenzoate (Ditty and Harwood 2002), and protocatechuate (Harwood and Parales 1996). Transport of phenylacetic acid has been found to take place via a similar mechanism in *Pseudomonas putida* U (Schleissner, Olivera et al. 1994) wherein PhaK is a porin that forms an outer membrane channel with a high degree of substrate specificity while PhaJ is an inner membrane MFS permease, also with high specificity (Olivera, Minambres et al. 1998). It was demonstrated that in *P. putida* U, transport is most likely coupled to electrochemical gradients and that the membrane is impermeable to phenylacetic acid. However, the membrane characteristics of *P. putida* U are considered highly unusual in that *P. putida* U is impervious to membrane disruption by fatty acids (Schleissner, Olivera et al. 1994). Therefore it is not clear that this transport system is representative of phenylacetic acid transport in general.

Despite its transport through a porin in *Pseudomonas putida* U, phenylacetate metabolism and regulation have been shown in recent years to perhaps be more akin to that of fatty acids than aromatic compounds. For example, phenylacetate is ligated to coenzyme-A as a prerequisite for further metabolism and the CoA ligate serves as a genetic regulator for downstream metabolic elements (Galan, Garcia et al. 2004).

A second type of transport system, the ABC transporter, has been found to be utilized in the uptake of certain aromatic acids. ABC (short for ATP-binding cassette) transporters are multi component active uptake systems unlike the MFS permease symporters. Whereas MFS proteins are porins, ABC systems are not (Busch and Saier 2002). ABC systems have been found to be utilized for the uptake of 3-hydroxyphenylacetate (Arias-Barrau, Sandoval et al. 2005) and phthalate (Hara, Stewart

et al. 2009). It is not clear why drastically different types of uptake strategies should be in use for the transport of such similar chemicals.

Because ibuprofen is nominally an aromatic acid, one may hypothesize that it is likely to be transported using an MFS porin or an ABC transporter. However, it is not a foregone conclusion that the aromatic ring is the main feature dictating the type of transport system employed. From a metabolic perspective, the aromatic ring poses a unique challenge. The dispersed electron cloud of the aromatic ring is particularly resistant to chemical modification. Entirely unique systems of catabolic enzymes have evolved specifically to tackle this challenge. On the other hand, from the perspective of transport across biological membranes, the aromatic ring is of little significance since it is simply a slightly bulky hydrophobic group which should have little trouble traversing the membrane. It is therefore possible that other structural characteristics will contribute to the selection of transport systems; while ibuprofen is an aromatic acid, it also has a long hydrophobic tail which includes the aromatic ring and the isobutyl-group, making it drastically more hydrophobic than any of the chemicals listed above.

Fatty acids are transported in an entirely different manner than aromatic acids. No inner-membrane spanning pores are required, nor is transport coupled directly to any ion gradients (Black and DiRusso 2003). This is most likely due to the tendency for fatty acids to partition into biological membranes due to their surfactant qualities. This characteristic makes for a chemical that can partition into and out of biological membranes, such that the membrane only slows diffusion to some degree but cannot prevent it. This presents a bacterium seeking to utilize the fatty acid with an entirely different situation from that faced by those seeking to utilize phenylacetate. In the case

of phenylacetate, the challenge lies in getting the molecule into the cell, whereas with fatty acids which freely diffuse across the membrane in both directions, the challenge lies in keeping the molecule inside the cell.

The transport approach that has evolved in the case of fatty acids which is found across all domains of life can be generally termed "vectorial ligation." (Black and DiRusso 2003; Zou, F. et al. 2003). The fatty acid, which freely passes into the cell down its concentration gradient, is ligated to coenzyme A, a bulky hydrophilic cofactor, a process that serves two transport purposes; the fatty acid is now trapped inside the cell because the CoA group cannot cross the membrane and the intracellular concentration of the fatty acid is effectively kept low, thus maintaining a concentration gradient and insuring continued influx (Weimar, DiRusso et al. 2002). It is actually this fatty acid CoA ligate that then typically serves as an inducer/repressor of regulation and as substrate a for further catabolism (Black, Faegerman et al. 2000; Campbell and Cronan 2001; DiRusso and Black 2004).

While the fact that vectorial ligation is a driving force in the uptake of fatty acids is at this point uncontestable, there is mounting evidence that there are additional transport mechanisms in place that enhance uptake. FadL is an outer membrane spanning protein that is found in many bacteria (van den Berg, Black et al. 2004; Hearn, Patel et al. 2008). FadL has high affinity for fatty acids and is proposed to translocate fatty acids via an accessory protein (TonB) driven by conformational change (Ferguson and Deisenhofer 2002; van den Berg, Black et al. 2004). Intriguingly, the transport of toluene by two other TonB-dependent members of the FadL family has been demonstrated in the cases of TbuX (Kahng, Byrne et al. 2000) and TodX (Wang, Rawlings et al. 1995).

The aliphatic side chain of ibuprofen that renders it much more hydrophobic than phenylacetic acid (K_{ow} 3.9 vs 1.4 (Hansch, Leo et al. 1995; Tixier, Singer et al. 2003)). This hydrophobic nature implies that it will have a much greater capacity to passively diffuse across the membrane, reducing the requirement for channel proteins such as the major facilitators and porins that have been observed to be involved in the transport of more hydrophilic aromatic acids. Chapter four of this dissertation details preliminary evidence suggesting that, like long chain fatty acids, ibuprofen transport proceeds via vectoral ligation, is dependent upon a CoA ligase, and does not rely upon a specific transmembrane transport system, though consideration of fatty acid and toluene transport systems suggest that further unidentified proteins may be involved.

Conclusions

The fate of ibuprofen in the environment is a poorly understood phenomenon. Given the overall lack of experimental evidence upon which to solidly base a hypothesis regarding how ibuprofen and related NSAIDs may be metabolized by environmental microbes, a direct experimental approach using classical microbiological methods was undertaken. By isolating an organism capable of growth on ibuprofen and studying the chemistry and genetics of the phenomenon we hoped to arrive at a solid understanding of said mechanism and to therefore make a solid contribution to the environmental sciences and microbiology.

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CHAPTER 2

FORMATION OF CATECHOLS VIA ACID SIDE CHAIN REMOVAL FROM IBUPROFEN AND RELATED AROMATIC ACIDS[1]

Abstract

Although ibuprofen (2-(4-isobutylphenyl)-propionic acid) is one of the most widely consumed drugs in the world, little is known regarding its degradation by environmental bacteria. *Sphingomonas* Ibu-2 was isolated from a wastewater treatment plant based on its ability to use ibuprofen as a sole carbon and energy source. A slight preference towards the R-enantiomer was observed, though both ibuprofen enantiomers were metabolized. A yellow color, indicative of *meta*-cleavage, accumulated in the culture supernatant when Ibu-2 was grown on ibuprofen. When and only when 3-flurocatechol was used to poison the *meta*-cleavage system, isobutylcatechol was identified in the culture supernatant via GC/MS analysis. Ibuprofen-induced washed cell suspensions also metabolized phenylacetic acid and 2-phenylpropionic acid to catechol, while 3- and 4-tolylacetic acids and 2-(4-tolyl)-propionic acid were metabolized to the corresponding methyl catechols before ring cleavage. This data suggests that in contrast to the widely distributed coenzyme A ligase, homogentisate, or homoprotocatechuate pathways for metabolism of phenylacetic acids, Ibu-2

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removes the acidic side chain of ibuprofen and related compounds prior to ring-cleavage.

Introduction

Ibuprofen (2-(4-isobutylphenyl-propionic acid)) is a pharmaceutical drug used for its analgesic, antipyretic, and anti-inflammatory properties. It is the third most consumed drug in the world, with an estimated annual production of several kilotons (Buser, Poiger et al. 1999). Approximately 10% of the ibuprofen consumed by humans is excreted unmodified or as the glucuronide conjugate (Lee, Williams et al. 1985; Rudy, Knight et al. 1991). Wastewater treatment processes have been found to remove ibuprofen with varying success, which may explain why Kolpin *et al.* (Kolpin, Furlong et al. 2002) detected ibuprofen in 9.5% of the bodies of water that they surveyed. Environmental concentrations of ibuprofen have been found to range from low part-per-trillion (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002) to low part-per-billion levels (Buser, Poiger et al. 1999; Farre, Ferrer et al. 2001).

Little information exists regarding how ibuprofen is oxidatively metabolized by environmental microbes. Side chain hydroxylation has been reported (Kolpin, Furlong et al. 2002; Zwiener, Seeger et al. 2002), along with the formation of carboxyhydratropic acid (2-(4-carboxyphenyl)propionic acid) (Buser, Poiger et al. 1999; Zwiener, Seeger et al. 2002) and ibuprofenol (2-(4-isobutylphenyl)-propanol) under anaerobic conditions (Chen and Rosazza 1994).

The most similar compounds whose bacterial metabolism have been described include 4-isopropylbenzoate (cumate), phenylacetic acid (PAA), and 2-phenylpropionic acid (2PPA). Cumate is dioxygenated at the 2,3 position by *Pseudomonas putida* F1 carrying the *cmt* operon and subsequently *meta*-cleaved (Defrank and Ribbons 1976; Defrank and Ribbons 1977; Eaton 1996; Eaton 1997). Other routes for the metabolism of phenylacetic acids include the well-characterized homoprotocatechuate (Sparnins and Chapman 1976) and homogentisate pathways (van den Tweel, Smits et al. 1988), as well as the more recently described CoA-ligase pathway (Ismail, Mohamed et al. 2003). Additionally, *Streptomyces rimosus* has been shown to convert 2PPA to 4-hydroxy-2PPA (Kuge, Mochida et al. 1991). Finally, *Pseudomonas cepacia* has been shown to metabolize 2PPA (2) and tropic acid (21) (2-phenyl-3-hydroxypropionic acid) via a pathway involving decarboxylation to phenylacetaldehyde followed by oxidation to PAA (Andreoni, Baggi et al. 1992).

In this study we report upon the isolation of a bacterium capable of utilizing ibuprofen as a sole carbon and energy source. We also provide evidence of an apparently novel metabolic pathway for the degradation of ibuprofen and related PAAs.

Methods

Materials and Strains

NAD^+ was purchased from SigmaAldrich (St. Louis, MO). Dextrin 10 was purchased from Fluka BioChemika (Buchs, Switzerland). All other chemicals were purchased from Acros (Morris Plains, NJ). A 50/50 enantiomeric mixture of *R/S*-

ibuprofen was used unless stated otherwise. Mineral salts medium (MSM) was prepared as previously described (McCullar 1994).

Isolation of Ibu-2 via enrichment of sewage sludge

Sewage sludge was enriched with ibuprofen according to standard protocols (Krieg 1981). A single colony was isolated and designated strain Ibu-2. A fragment of the 16S rRNA gene from Ibu-2 was PCR amplified using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1055R (CGGCCATGCACCACC) (Lane 1991) and was sequenced using the 27F and 1055R primers.

Stereospecificity

Ibu-2 was inoculated into 500 mg/L *R/S*-ibuprofen, 500 mg/L *S*-ibuprofen, or 250 mg/L *R/S*-ibuprofen. Pure *R*-ibuprofen was not used because it is not commercially available. Maximal cell density was determined spectrophometrically.

Chiral capillary electrophoresis (CE) was performed on the supernatants of ibuprofen grown Ibu-2 cultures in order to determine if a difference existed in the rate at which the enantiomers were metabolized. Supernatants were harvested and filtered as described above. Samples were run on an HP3D CE using a 40 cm x 50 μm fused silica column from Agilent technologies (Palo Alto, CA) and a method adapted from Simo *et al.* (Simo 2002). Cassette temperature was set at 25 ºC. The running buffer was composed of 6% dextrin 10 and 150 mM sodium borate at pH 9. Prior to each injection, the column was preconditioned for 1 min with water, 1 min with 0.1 M NaOH and 50 mM SDS, and finally for 2 min with the running buffer. Injection was performed by

applying 10 mbar pressure for 9 seconds followed by running buffer injection at 50 mbar for 5 seconds. Voltage was applied at +20 kV. A diode array detector was used with detection and reference wavelengths set at 194 +/- 2 nm and 500 +/- 80 nm respectively.

Substrate specificity analysis with washed cells

Ibu-2 was grown in MSM containing 500 mg/L ibuprofen or 0.1% glucose. Washed cell suspensions (WCS) were prepared according to standard protocols (Focht 1994). Test compounds were added to a final concentration of 500 mg/L in 500 µl aliquots of WCS and yellow color generation was monitored for thirty min. The yellow products were further characterized by determining their absorbance maxima via spectrophotometry. Yellow color was assumed to be indicative of *meta*-cleavage product and was thus taken as positive indication of metabolism of the test compound.

Chemicals tested included phenol, 2-, 3-, and 4-methylphenol, catechol, 3-methylcatechol, 4-methylcatechol, 4-tertbutylcatechol, benzoate, 4-methylbenzoate, phenylacetaldehyde, phenylacetic acid, *R*- and *S*-2-hydroxy-2-phenylacetic acid (mandelic acid) 2-, 3-, and 4-tolylacetic acid, 2- and 4-(hydroxyphenyl)acetic acid, 2- and 3-phenylpropionic acid, 2-(4-tolyl)propionic acid, 2-phenyl-3-hydroxypropionic acid (tropic acid), 3-phenyl-2-propenoic acid (cinnamic acid), 2-phenylbutyric acid, and 2,2-diphenylacetic acid.

Growth substrate analysis

Growth of Ibu-2 was tested on compounds that gave a positive result in the substrate specificity analysis. Tests were performed in triplicate in test tubes. 5 ml of an

overnight Ibu-2 culture grown on ibuprofen was used to inoculate MSM containing 250 mg/L or 500 mg/L of the chemical of interest. The tubes were placed on a vertical rotor and monitored over the course of one week for growth via changes in OD at 600 nm (OD_{600}).

Compounds that did not support growth on their own were assayed for their ability to support growth in the presence of ibuprofen as an inducer. Ibu-2 was inoculated into mixtures of 250 mg/L of these compounds plus 250 mg/L ibuprofen in MSM.

Analysis of culture supernatants by GC/MS

Ibu-2 was inoculated into 1 L of MSM containing 500 mg of ibuprofen. When grown on ibuprofen, the culture accumulated a yellow color. The culture was allowed to continue growing until that color reached an apparent maximum level (48-60 hours). At this point, the supernatant was harvested via centrifugation and filtered through a 0.22 μm filter. The supernatant was then acidified to pH 3 using 1 M HCl and extracted with 50 ml of ethyl acetate. The extract was concentrated to a volume of 2 ml under nitrogen at 35°C. The samples were then methylated with diazomethane by standard protocols (Hecht and Kozarich 1972). After thirty min at room temperature, the samples were evaporated to a minimal volume under a nitrogen stream and analyzed via GC/MS using an HP 6890 GC equipped with an HP-5MS column (5% phenyl methyl siloxane 30 m x 0.25 mm, 0.25 μm film thickness) using helium as the carrier gas with a flow rate of 1 ml/min. The injector temperature was 250°C. The initial oven temperature of 40°C was held for 1 min, then ramped at a rate of 10°C/min to 250°C. The temperature was held at

250°C for 7 min and then ramped up at 30°C/min until 300°C. The detector was an HP 5973 MSD with the quadrupole and source set at 150ºC and 230ºC respectively.

Analysis of catecholic ibuprofen intermediates

3-fluorocatechol, a *meta*-cleavage inhibitor, was added to a final concentration of 50 mg/L in 100 ml of mid-log-phase Ibu-2 culture. After 30 min, the supernatant was removed and filtered. Potassium carbonate and acetic anhydride were added to final concentrations of 1.5% and 0.5% respectively. This aqueous acetylation was performed to selectively acetylate aromatic hydroxyl groups (Mars 1997). After thirty min, the samples were acidified to pH 3, extracted with ethyl acetate, and dried over a sodium sulfate column. The extracts were then methylated with diazomethane and analyzed as described above. A culture with ibuprofen but without 3-fluorocatechol was also analyzed.

Characterization of catechols produced from ibuprofen analogs

Assays were performed with WCS and the 2-arylpropionic acids and phenylacetic acids that produced yellow metabolites. The assays were performed with and without the addition of 3-fluorocatechol. The supernatants were passed through a 0.22 μm filter and then analyzed via HPLC for accumulation of catechols. The HPLC retention times of the metabolites were compared to those of catechol and methylcatechol standards. HPLC was performed using 70% methanol, 30% 40 mM acetic acid as the eluent. The sample was pumped at a rate of 1 ml/min using a Waters Model 590 pump through a Varian Microsorb-MV C18 column (250 mm by 4.6 mm). Samples were injected by a Shimadzu

SIL-10AD AP autoinjector and detected with a Shimadzu SPD-10A VP UV-Vis detector by monitoring absorbance at 285 nm.

Cell-free extract assays

Ibuprofen-grown Ibu-2 was washed with 0.9% NaCl to remove the exopolysaccharide matrix (Richau 2000) and then resuspended in a minimal volume of sonication buffer (100 mM tris, 2 μm phenylmethylsulfonyl fluoride, and 1 μm dithiothreitol at pH 8 (Long 1997)). The cells were then lysed via sonication using a Branson Sonifer 450 set at 100% duty cycle using three cycles of 30 seconds each separated by 1 min rest time. The cells were kept on ice at all times. The samples were then centrifuged at 21000 x g at 4°C for 15 min and the supernatant retained. Extracts were assayed for activity by adding catechol to a small aliquot and monitoring for yellow product generation.

Assaying sidechain oxidation

Cell extracts from *Pseudomonas* AT3 grown on tropic acid, prepared using the method described above, were used as a positive control for side chain oxidation (Long 1997). The assay, which monitors NAD^+/$NADP^+$ reduction, was performed with cell free extracts from Ibu-2 or AT3 in sonication buffer with either 0.1 μM tropic acid or ibuprofen and 0.1 μM of either NAD^+ or $NADP^+$. The reactions were incubated at room temperature and monitored for reduction of NAD^+ or $NADP^+$ at 340 nm.

Results and Discussion

Ibu-2 grew on ibuprofen as a sole source of carbon and energy (data not shown). When grown on ibuprofen in liquid media, a yellow color appeared in the supernatant. This yellow color disappeared upon acidification and reappeared upon neutralization, a phenomenon diagnostic of *meta*-cleavage products (*mcp*). Sequencing and BLAST analysis (Altschul 1990) of the 16S rRNA gene fragment revealed that Ibu-2 was 98% identical to *Sphingomonas* species over 967 bp. Ibu-2 had yellow pigmentation and tended to develop an exopolysaccharide matrix, especially when grown on glucose. Both of these characteristics are common to *Sphingomonas* species (Pollock 1993).

Stereospecificity

Ibu-2 grew to the same maximum cell density on 500 mg/L *R/S*-ibuprofen as it did on 500 mg/L *S*-ibuprofen. Both of these values were approximately twice that obtained using 250 mg/L *S* ibuprofen or *R/S*-ibuprofen. During growth on *R/S*-ibuprofen the entantiomeric fraction dropped to less than 35% *R*-ibuprofen before both isomers where completely removed, suggesting that Ibu-2 may preferentially degrade the *R* enantiomer (Figure 2.1).

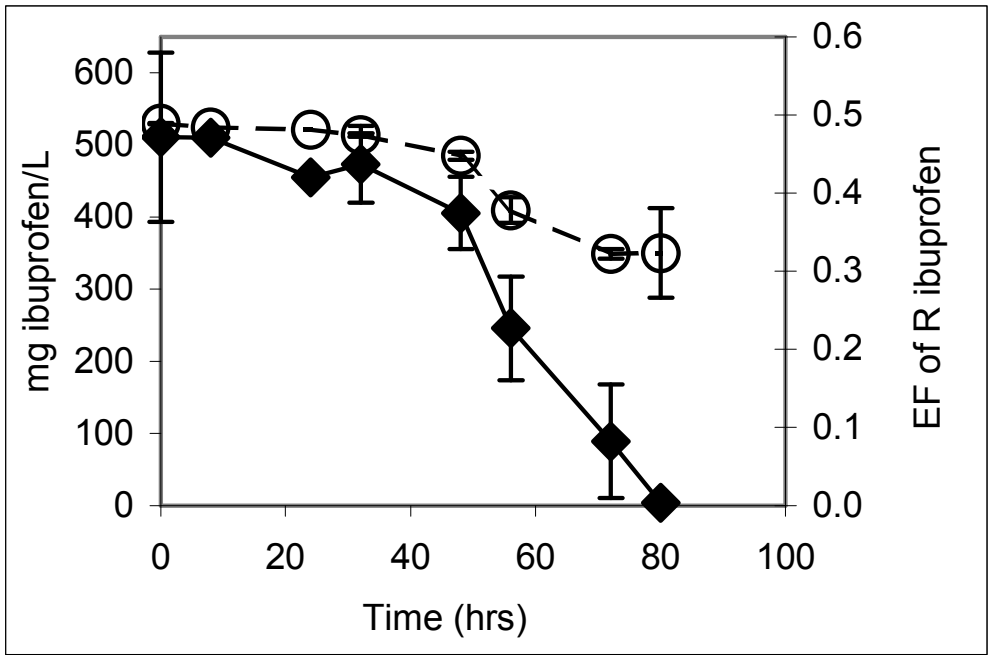

Figure 2.1. The enantiomeric fraction (EF) of R-ibuprofen (-○-) and overall ibuprofen concentration (-♦-) in a growing Ibu-2 culture in which ibuprofen is the sole carbon and energy source. The concentrations of both ibuprofen enantiomers were determined by chiral CE analysis.

GC/MS analysis of culture supernatant extracts

When ibuprofen-grown cultures were poisoned with 3-fluorocatechol, a metabolite accumulated in the supernatant whose mass spectrum was consistent with isobutylcatechol (metabolite B, Table 2.1). The mass spectrum of this compound revealed a molecular ion at *m/z* 250, which is consistent with diacetylated isobutylcatechol. The two acetyl groups, which were added during aqueous acetylation, are diagnostic of the presence of two aromatic hydroxyl groups (Mars 1997). Acetyl groups give predictable losses of *m/z* 42, which in this case accounted for the peaks at *m/z* 208 and 166. The other large peak at *m/z* 123 represents a loss of 43 which is consistent with the loss of the isopropyl group from the base ion fragment. The combination of this mass spectral fragmentation pattern, the derivatizable nature of metabolite during

aqueous acetylation, and its accumulation only in the presence of 3-fluorocatechol are strong evidence that the peak detected via GC-MS was indeed isobutylcatechol.

Mass spectra were also obtained for two other compounds from the extract of a culture that was not poisoned with 3-fluorocatechol and which had accumulated high levels of *mcp* (metabolites C and D, Table 2.1).

Table 2.1. GC/MS retention times and major ions of isobutylcatechol (B) and two putative isobutylcatechol meta-cleavage metabolites (C and D).

MS	ret. time (min.)	mass (relative abundance)				
b	15.7	123(99)	166(100)	208(17)	250(4)	
c	14.7	226(2)	167(100)	151(6)	137(1)	123(8)
d	15.8	256(10)	225(10)	197(100)	139(20)	

These spectra were not detected in the extract of cultures poisoned with 3-fluorocatechol. One spectrum was consistent with the *mcp* of isbutylcatechol: 5-formyl-2-hydroxy-7-methylocta-2,4-dienoic acid (metabolite C, figure 2.2), and the other was consistent with its formyl-oxidized derivative, 2-hydroxy-5-isobutylhexa-2,4-dienedioic acid (metabolite D, figure 2.2).

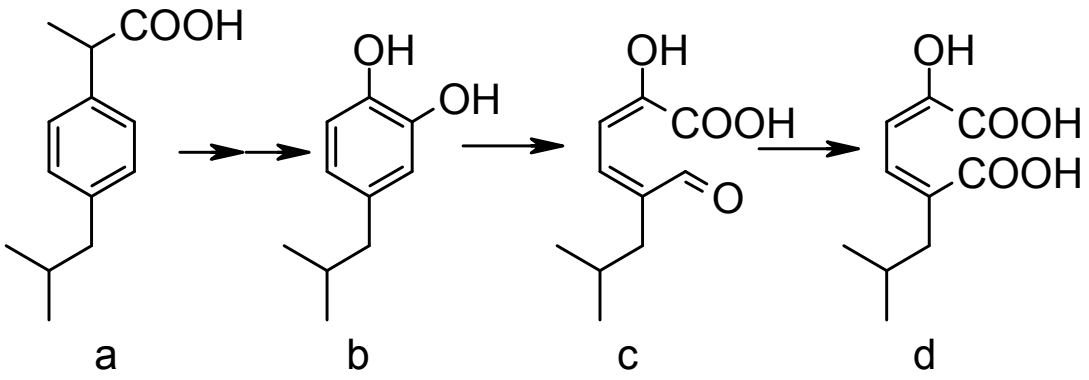

Figure 2.2. Proposed pathway for the metabolism of ibuprofen by Ibu-2. Metabolites b-d were all detected via GC/MS. b: isobutylcatechol; c: 5-formyl-2-hydroxy-7-methylocta-2,4-dienoic acid; d: 2-hydroxy-5-isobutylhexa-2,4-dienedioic acid

As expected, treatment with diazomethane methylated both the acidic hydroxyl and the alpha carbon hydroxyl groups of metabolites C, giving a molecular ion of *m/z* 226. The *m/z* 167 fragment represents a loss of 59 from the parent ion, which is consistent with the loss of a methylated carboxyl group and is a common loss from aliphatic esters. The *m/z* 137 fragment is consistent with the loss of CH_2O from the *m/z* 167 fragment.

The major fragments of metabolite D (*m/z* 256 / 225 / 197 / 139) are consistent with the expected transformation product of metabolite C. After derivatization this would be expected to have three additional methyl groups, one on each acidic hydroxyl group and one on the *alpha* hydroxyl group. A loss of 31 to give *m/z* 225 is consistent with loss of CH_2OH from the parent ion. An alternative loss from the parent ion (*m/z* 256) yielded a fragment with an *m/z* 197 and is consistent with the loss of a methylated carboxylic acid group (-59). Further impact of this fragment would be expected to result in a loss of 58 which would correspond to removal of the second methylated carboxylic acid group and yield the fragment with an *m/z* 139.

Substrate specificity analysis

Ibu-2 washed cell suspension was also able to metabolize phenylacetic acid, 3- and 4-tolylacetic acids, 2-phenylpropionic acid, and 2-(4-tolyl)-propionic acid. However, it was not able to metabolize 2-phenylbutyric acid or 2,2-diphenylacetic acid, which implies that the nature of the substitution on the alpha carbon is important. Neither phenol nor any methylphenol was metabolized, making it less likely that a phenolic metabolite was involved. Washed cell suspension could not metabolize either mandelic acids or tropic acid (2-phenyl-3-hydroxypropionic acid), implying that hydroxylation of the acid side chain was not an intermediate step in side-chain removal. Furthermore, washed cell suspension was not able to metabolize benzoate or 4-methylbenzoate, suggesting that benzoic acids, which have been shown to be intermediates in the anaerobic degradation of phenylacetic acid by a *Pseudomonas* sp. and by *Azoarcus evansii* (Mohamed, Ismail et al. 2002), were not likely to be intermediates in ibuprofen degradation. Other compounds that were not metabolized include 4-tertbutylcatechol, phenylacetaldehyde, 2-tolylacetic acid, 2- and 4-(hydroxyphenyl)acetic acid, 3-phenylpropionic acid, 3-phenyl-2-propenoic acid (cinnamic acid). In all cases, it is possible that lack of metabolism was due to lack of transport into the cell.

Growth substrate analysis

The only ibuprofen analogs that supported growth of Ibu-2 without the presence of ibuprofen as an inducer were 3-tolylacetic acid and 2-(4-tolyl)propionic acid. However, 4-tolylacetic acid was able to support growth when ibuprofen was also present in the media. Phenylacetic acid and 2-phenylpropionic acid did not support growth under

any conditions (Figure 2.3). Surprisingly, 3-tolylacetic acid supported less than half of the growth permitted by ibuprofen or 4-tolylacetic and 4-tolylpropionic acids ($p < 0.02$). This suggests the possibility of toxicity or incomplete metabolism. If the latter were true, accumulation of a metabolite might have been predicted. However, no dead-end metabolites were detected when the culture supernatant from 3-tolylacetic acid grown cells was subjected to HPLC or GC/MS analysis (data not shown).

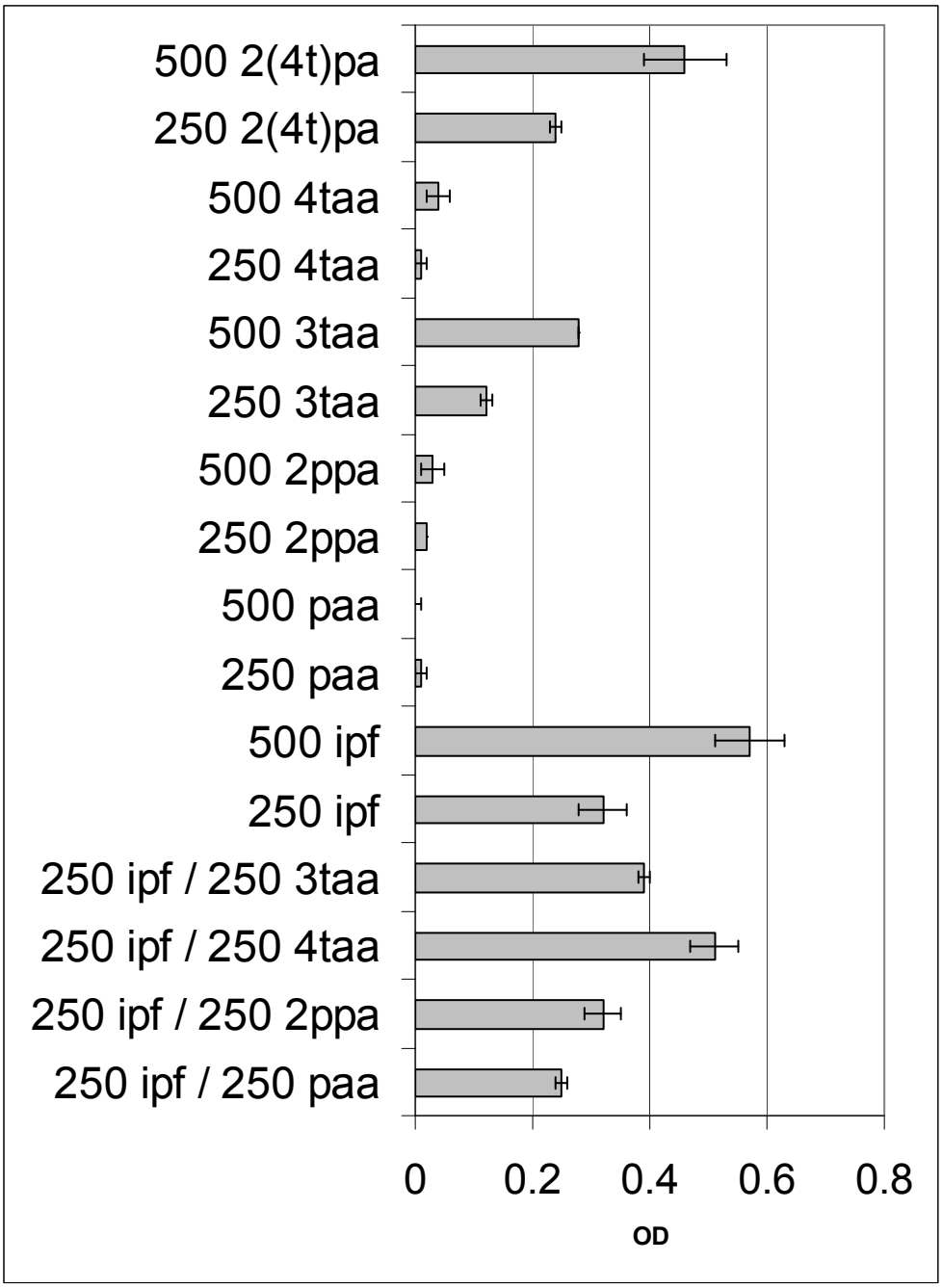

Figure 2.3. Average final culture density (n=3) as measured by OD_{600} when Ibu-2 was inoculated in liquid MSM culture containing ibuprofen or its analogs (ipf ibuprofen, paa phenylacetic acid, 2ppa 2-phenylpropionic acid, 3taa 3-tolylacetic acid, 4taa 4-tolylacetic acid, 2(4t)pa 2-(4-tolyl)propionic acid).

Characterization of catechols produced from ibuprofen analogs

When the supernatants of WCS that had been incubated with ibuprofen analogs and 3-fluorocatechol were examined via HPLC, each exhibited a novel peak. These peaks were only present after the addition of 3-fluorocatechol. In the phenylacetic acid and 2-phenylpropionic acid samples, a peak appeared whose retention time matched that of catechol (6.4 min). Within 2 hrs, almost 60% of the phenylacetic acid added was converted to catechol and no other intermediates were detected, suggesting that the observed deacylation was not merely an unproductive side reaction. In the 3-tolylacetic acid sample, a peak appeared whose retention time matched that of 3-methylcatechol (13.5 min). In the 4-tolylacetic acid and 2-(4-tolyl)-propionic acid samples, a peak appeared whose retention time matched that of 4-methylcatechol (11.6 min).

Characterization of meta-cleavage products of ibuprofen analogs.

The *mcps* of phenylacetic acid, 2-phenylpropionic acid, and catechol all had the same maximum absorbance wavelength (378 nm). The *mcps* of 3-tolylacetic acid and 3-methylcatechol also had the same maximum absorbance wavelength (380 nm), as did 4-tolylacetic acid, 2-(4-tolyl)-propionic acid, and 4-methylcatechol (384 nm). These observations all suggest that deacylation of the acidic side chain occurred before ring cleavage.

Cell-free extract activities upon ibuprofen

Ibu-2 cell-free extracts readily produced *mcp* from catechol, 3-methylcatechol, and 4-methylcatechol (data not shown). Ibu-2 cell-free extracts did not generate any

yellow color when incubated with ibuprofen with or without the addition of NADH/ferrous iron, nor was any ibuprofen disappearance detected via HPLC under any conditions. The Ibu-2 cell free extracts did not reduce NAD^+ or $NADP^+$ in the presence of either ibuprofen or tropic acid, although the positive control reduced NAD^+ in the presence of tropic acid. Cofactor reduction woμld have been expected if Ibu-2 used a *Pseudomonas* AT3-like mechanism to oxidize the sidechain of ibuprofen.

Conclusions

Unlike *Variovorax* sp Ibu-1, which has been suggested to dioxygenate the aromatic ring of ibuprofen in the 2 and 3 positions (Murdoch 2002), Ibu-2 appears to metabolize ibuprofen through a novel mechanism resμlting in removal of the propionic acid moiety and dioxygenation of the ring at the 1,2 position, giving rise to isobutylcatechol. The accumμlation of this compound when and only when a *meta*-cleavage inhibitor was added suggests that isobutylcatechol is further metabolized via *meta*-cleavage. The identification of compounds consistent with the *meta*-cleavage of isobutylcatechol only when a *meta*-cleavage inhibitor was not added lends further support to this conclusion.

As a similar side chain removal of related 2-phenylpropionic acids or phenylacetic acids has not been previously reported, the ability of Ibu-2 to metabolize other aromatic acids was determined. The production of catechols and their respective *mcps* from these compounds provides additional evidence for this unique pathway.

The exact mechanism whereby Ibu-2 accomplished this acid side chain removal remains to be determined. Although it is likely that other steps were required to activate the acid moiety prior to removal, we could not detect any evidence suggesting the involvement of other intermediates prior to the formation of catechols. Further work will be required before the mechanism whereby this is accomplished can be elucidated.

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CHAPTER 3

GENETIC AND CHEMICAL CHARACTERIZATION OF IBUPROFEN AND PHENYLACETIC ACID METABOLISM BY *SPHINGOMONAS* IBU-2

Abstract

Sphingomonas Ibu-2 has the unique ability to cleave the acid side chain from the non-steroidal anti-inflammatory drug ibuprofen and related phenylacetic acid derivatives to yield corresponding catechols under aerobic conditions. To identify the genes involved in this unusual pathway, we constructed a chromosomal library of Ibu-2 DNA in *Escherichia coli* epi300. Fosmid clones capable of metabolizing ibuprofen to isobutylcatechol were identified. Transposon mutagenesis of one of the fosmids (pFOS3G7) allowed identification of thirteen mutants that failed to produce detectatable catechols. DNA sequencing revealed insertions in five open reading frames *ipfABDEF* whose predicted amino acid sequences bore similarity to the large and small units of aromatic dioxygenases, an SCPx thiolase, a domain of unknown function 35 (DUF35), and an aromatic coenzyme A ligase respectively. A knockout of *ipfH* which putatively encodes a ferredoxin reductase component of an aromatic dioxygenase system showed reduced catecholic metabolite production. An open reading frame *ipfI* which putatively encodes the ferredoxin component of an aromatic dioxygenase was also

identified on pFOS3G7. Complementation of a markerless loss-of-function *ipfD* deletion mutant with *ipfD* restored catechol-generation ability. Subcloned *ipfABDEF* produced catecholic metabolites, but only in trace amounts. The co-expression of *ipfABDEF* with *ipfH* and *ipfI* greatly enhanced the production of catecholic metabolites. The existence of the isobutylcatechol metabolite and the novel involvement of an SCPx thiolase (IpfD) and a DUF35 protein (IpfE) in the generation of the catechol suggest that this pathway is distinct from others described to date and represents a novel aerobic paradigm employed for the metabolism of alkyl aromatic acids.

Introduction

Ibuprofen (2-(4-isobutylphenyl-propionic acid)) is a pharmaceutical with analgesic, antipyretic, and anti-inflammatory properties. With an annual production of several kilotons (Buser, Poiger et al. 1999), it is the most widely used member of a diverse class of pharmaceuticals termed the non-steroidal anti-inflammatory drugs (NSAIDs), many of which share a phenylacetic acid (PAA) core such as diclofenac, naproxen, ketoprofen, and flurbiprofen (Figure 1.1). Approximately 10% of the ibuprofen consumed by humans is excreted unmodified or as the glucuronide conjugate (Lee, Williams et al. 1985; Rudy, Knight et al. 1991). The drug's popularity, large therapeutic dose, and incomplete human metabolism lead to a large amount of ibuprofen entering the environment with human waste. Wastewater treatment processes have been found to remove ibuprofen to varying degrees (Buser, Poiger et al. 1999; Stumpf, Ternes

et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002; Fent, Weston et al. 2006; Benotti, Trenholm et al. 2008; Huerta-Fontela and Ventura 2008; Corcoran, Winter et al. 2010; Kummerer 2010; Santos, Araújo et al. 2010) which may explain why ibuprofen has been detected in bodies of water all over the world (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002). Ibuprofen has also been detected in water used for irrigation (Pedersen, Yeager et al. 2003; Pedersen, Soliman et al. 2005; Kinney, Furlong et al. 2006; Siemens, Huschek et al. 2008; Xu, Wu et al. 2009) and municipal drinking supplies (Jones, Lester et al. 2005). Environmental concentrations of ibuprofen have been found to range from low part-per-trillion (Buser, Poiger et al. 1999) to low part-per-billion levels (Buser, Poiger et al. 1999; Farre, Ferrer et al. 2001; Santos, Araújo et al. 2010)

Investigations into subtle effects and mixture toxicity have found ibuprofen induced changes on the timing of spawning by medaka (Flippin, Hugget et al. 2007; Han, Choi et al. 2010), growth/predominance of algae and duckweed (Pomati, Netting et al. 2004; Richards, Wilson et al. 2004), microbial diversity in aquatic mesocosms (Richards, Wilson et al. 2004), and riverine biofilm communities (Lawrence, Swerhone et al. 2005) at environmentally relevant concentrations.

Little information exists regarding how ibuprofen is oxidatively metabolized by environmental microbes. The fungus *Verticillum lecanii* has been shown to hydroxylate ibuprofen on the isobutyl group (Hanlon 1994) and similar microbially-generated hydroxyibuprofen metabolites along with carboxylated ibuprofen have been detected (Zwiener, Seeger et al. 2002; Quintana, Weiss et al. 2005; Marco-Urrea, Pérez-Trujillo et

al. 2009). However, these metabolites have not been linked directly with the ability to use ibuprofen as a growth or energy source nor did they account for the majority of the added ibuprofen.

Sphingomonas Ibu-2 is able to use racemic ibuprofen as a sole carbon and energy source (Murdoch and Hay 2005). Ibu-2 utilizes a classical catechol *meta*-cleavage type pathway. However, the catechol that is generated from ibuprofen is isobutylcatechol, the creation of which requires the removal of the propionic acid side-chain from the aromatic ring (Figure 3.1). It was also demonstrated that Ibu-2 performs similar reactions with other phenylacetic acids including phenylacetic acid, 2-phenylpropionic acid, 3- and 4-tolylacetic acids, and 2-(4-tolyl)propionic acid, converting them to the corresponding catechols (catechol or methylcatechol).

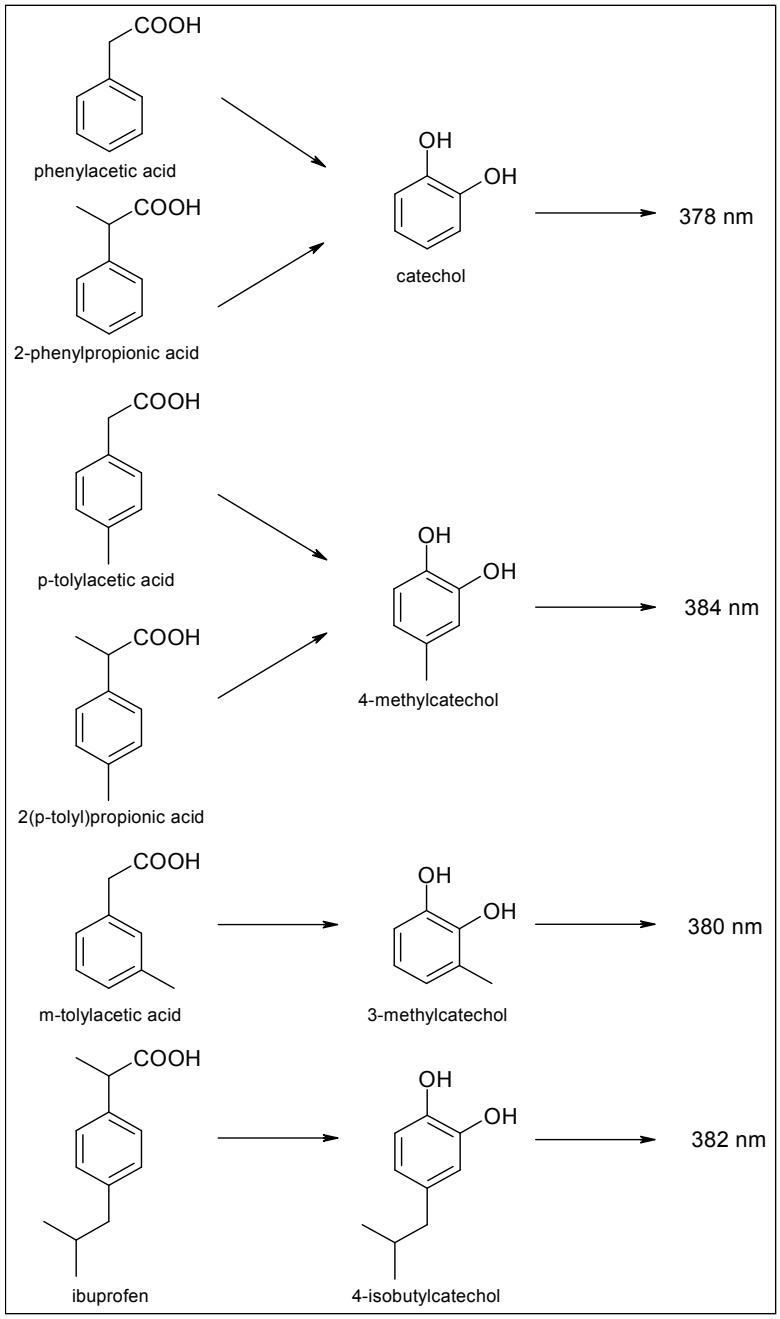

Figure 3.1. Catechols and corresponding meta-cleavage products absorbance maxima and the substrate chemicals as detected in ibuprofen-induced resting *Sphingomonas* Ibu-2 (Chapter 2).

There is to this author's knowledge no precedent for the ejection of an acyl group from an aromatic ring in such a manner, though it is reminiscent of 1,2- benzoate dioxygenases that remove the carboxyl moiety from benzoate and which have been extensively

described (Reiner 1971; Fetzner, Muller et al. 1992; Jeffrey, Cuskey et al. 1992; Eaton 1996). This side chain removal phenomenon also differs from published reports of the metabolism by other bacteria of phenylacetic acid which has been shown to take place via coenzyme A ligation followed by epoxidation and non-oxygenolytic ring cleavage but does not involve catecholic intermediates (Martinez-Blanco, Reglero et al. 1990; Mohamed 2000; Rost, Haas et al. 2002; Ismail, Mohamed et al. 2003; Fernandez, Ferrandez et al. 2006; Teufel, Mascaraque et al. 2010).

The metabolism of ibuprofen and related phenylacetic acids by *Sphingomonas* Ibu-2 appears to represent a new paradigm for the metabolism of phenylacetic acids. In order to identify the genes responsible for the conversion of these aromatic acids to the corresponding catechols, a fosmid library was constructed of Ibu-2 total DNA. A fosmid that conferred upon *E. coli* the ability to generate isobutylcatechol from ibuprofen was subjected to transposon mutagenesis. We report here on the results of these efforts and a description of the steps involved in ibuprofen and phenylacetic acid degradation by *Sphingomonas* Ibu-2.

Material and Methods

Materials

Unless otherwise noted, chemicals were purchased from Acros (Morris Plains, NJ). Luria-Bertani broth (LB) was prepared as previously described (Sambrook, Fritsch et al. 1989). Mineral salts medium (MSM) was prepared as previously described (McCullar 1994).

Creation and screening of Ibu-2 fosmid library

Ibu-2 chromosomal DNA was extracted from cells harvested from 100 mL of liquid MSM culture containing 500 mg/L ibuprofen (ipf) by the standard alkaline lysis procedure (Sambrook, Fritsch et al. 1989). The harvested DNA was quantified using the Ribogreen Kit (Molecular Probes, Eugene, OR). The DNA was prepared and the fosmid library was created according to the instructions in the CopyControl™ Fosmid Library Production Kit instructions (Epicentre Biotechnologies, Madison, WI). Briefly, the DNA was sheared and size selected for 35kb-45kb. The DNA was then end-repaired, ligated into the fosmid vector, and packaged in phage particles. These particles were then used to transfect *E. coli* epi300 and successful transformants were selected on LB 25 mg/L chloramphenicol (chl). A 900 clone library (approximately 7X expected genome coverage) was constructed in 96-well plates and stored with the addition of 20% glycerol at -80 deg C. The fosmid library was screened for the accumulation of dark brown catecholic polymers when grown in LB with 50 mg/L ibuprofen in 96-well plates. 10mM arabinose was used to induce the fosmid to high copy number in all metabolic assays.

Creation and metabolic screening of fosmid clone transposon libraries

The *EZ::TN* <TET-1> Insertion Kit (Epicentre Technologies, Madison, WI) was used to create transposon insertion mutants of chromosomal library fosmid pFOS3G7 which was positive for isobutyl catechol accumulation. The mutagenesis was performed as described in the kit instructions. The reaction was packaged in phage extract (MaxPlax Lambda Packaging Extract, Epicentre Technologies, Madison, WI), transfected

into *E. coli* epi300, and selected on LB plates containing 25 mg/L chl and 12 mg/L tetracycline (tet). A 96-clone library of transposon mutants was then screened for loss of the ability to accumulate the brown color associated with catechol production and polymerization.

The loss-of-function mutants were characterized by sequencing DNA surrounding the site of transposon insertion using transposon-specific primers FP and RP (Table 3.2). The sequence information was compiled using the SeqManager program (DNAStar, Inc., Madison, WI) and searched for open reading frames using the GeneQuest program (DNAStar, Inc., Madison, WI). Similarity to known and putative proteins was assessed using BLAST-P (Altschul 1990).

Table 3.1. Strains and plasmids used in this study.

Strains		
Sphingomonas Ibu-2	isolated from Ithaca, NY sewage treatment plant via enrichment for growth on ibuprofen	this study
E. coli S17	λpir; *hsdR pro recA*; RP4 2-Tc::Mu-Km::Tn7, pro, res-, mod+, streptomycin resistance, trimethoprim resistance	(Simon, Priefer et al. 1983)
E. coli JM109	recA1 subE44 endA1 hsdR17 gyrA96 relA1 thi Δ*(lac-proAB*) F' (*traD36 proAB+ lacIq lacZ ΔM15*)	(Sambrook, Fritsch et al. 1989)
E. coli epi300	F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG trfA tonA*	Epicentre, Madison, WI

Plasmids		
pKD4	contains the template for generating FLP recombinase target lambda red kanamycin resistance PCR fragment; kanamycin resistant	(Datsenko and Wanner 2000).
epi300 3G7 pKD46	arabinose-inducible lambda red recombinase expression plasmid; ampicillin resistant	(Datsenko and Wanner 2000).

pCP20	heat inducible FLP recombinase expression plasmid	(Datsenko and Wanner 2000).
pCC1FOS	copy control fosmid vector, inducible to high copy number with arabinose when hosted in *E. coli* epi300, chloramphenicol resistance	Epicentre, Madison, WI
pFOS3G7	pCC1FOS *Sphingomonas* Ibu-2 chromosomal library clone 3G7	this study
pFOS4F6	pCC1FOS *Sphingomonas* Ibu-2 chromosomal library clone 4F6	this study
pBBR1mcs	broad host range plasmid, chloramphenicol resistance	(Kovach, Elzer et al. 1995)
pJ25	pBBR1mcs with BamHI/NsiI fragment from pCC1FOS 3G7 bearing *ipfABDEF*	this study
pFOS3G7Tn:*ipfA*	pFOS3G7 with Tn5 insertion in *ipfA*, transposon library clone F1, chloramphenicol and tetracycline resistant	this study
pFOS3G7Tn:*ipfB*	pFOS3G7 with Tn5 insertion in *ipfA*, transposon library clone H6, chloramphenicol and tetracycline resistant	this study
pFOS3G7Tn:*ipfD*	pFOS3G7 with Tn5 insertion in *ipfD*, transposon library clone A2, chloramphenicol and tetracycline resistant	this study
pFOS3G7Tn:*ipfE*	pFOS3G7 with Tn5 insertion in *ipfE*, transposon library clone E6, chloramphenicol and tetracycline resistant	this study
pFOS3G7Tn:*ipfF*	pFOS3G7 with Tn5 insertion in *ipfF*, transposon library clone F10, chloramphenicol and tetracycline resistant	this study
pFOS3G7ΔipfA	pFOS3G7 with markerless deletion of ipfA, chloramphenicol resistant	this study
pFOS3G7ΔipfB	pFOS3G7 with markerless deletion of ipfB, chloramphenicol resistant	this study
pFOS3G7ΔipfD	pFOS3G7 with markerless deletion of ipfD, chloramphenicol resistant	this study
pFOS3G7ΔipfE	pFOS3G7 with markerless deletion of ipfF, chloramphenicol resistant	this study
pGEMt-easy	ampicillin resistance	Promega, Madison, WI
pGEM:*ipfA*rbs	pGEMt-easy with ipfArbsF/ipfAR PCR product ipfA including a three frame stop codon and a ribosomal binding site,	this study
pGEM:*ipfB*rbs	pGEMt-easy with ipfBrbsF/ipfBR PCR product ipfB including a three frame stop codon and a ribosomal binding site,	this study
pGEM:*ipfDE*rbs	pGEMt-easy with ipfDrbsF/ipfER PCR product ipfDE including a three frame stop codon and a ribosomal binding site,	this study
pGEM:*ipfD*rbs	pGEMt-easy with ipfDrbsF/ipfDR PCR product ipfD including a three frame stop codon and a ribosomal binding site,	this study

pGEM:*ipfE*rbs	pGEMt-easy with ipfErbsF/ipfER PCR product ipfE including a three frame stop codon and a ribosomal binding site,	this study
pGEM:*ipfAB*	pGEMt-easy with ipfAF and ipfBR PCR product ipfAB	this study
pGEM:*ipfF*	pGEMt-easy with ipfFF/ipfFR PCR amplicon ipfF	this study
pGEM:*ipfHI*	pGEMt-easy with *ipfHI* PCR amplicon created by strand overlap extension	this study

Functional analysis of pFOS3G7 clones via HPLC

Overnight cultures of *E. coli* epi300 harboring pFOS3G7 or different transposon mutants of pFOS3G7 were inoculated (10% v/v) into five ml of LB containing 50 mg/L ibuprofen, the appropriate antibiotics, and 10mM arabinose. The cultures were incubated at 37°C in a rotary shaker. One ml samples were taken at the initiation of the experiment and at four days and analyzed for ibuprofen concentration via HPLC. The HPLC solvent, 80% methanol 20% 40mM acetic acid, was pumped at a rate of 1 ml/min using a Waters Model 590 pump through a Varian Microsorb-MV C18 column (250 mm by 4.6 mm). Samples and standards were injected by a Shimadzu SIL-10AD AP autoinjector and detected with a Shimadzu SPD-10A VP UV-Vis detector. The UV-Vis signal was fed into a PC computer where it was collected and analyzed using Peaksimple (SRI Instruments, Torrance, CA). Ibuprofen was quantified by comparison to a standard curve.

GC/MS was performed on overnight cultures to characterize catecholic metabolites or other detectable metabolites that might accumulate. Aqueous acetylation was performed by previously described methods (Murdoch and Hay 2005) in order to selectively derivatize aromatic hydroxyl groups. The sample was then extracted with ethyl acetate and dried over a sodium sulfate column. The ethyl acetate extracts were

methylated using diazomethane and dried once more over a sodium sulfate column. GC/MS was performed using previously described methods (Murdoch and Hay 2005).

Presence or absence of catechols was determined by HPLC analysis of washed cell suspensions that had been concentrated 20-fold and was performed as previously destribed (Murdoch and Hay 2005).

Fosmid subcloning

Analysis of sequence from the transposon mutants implicated an approximately 5kb region of fosmid pFOS3G7 as being involved in the production of isobutylcatechol (Figure 3.4). This region was gel purified away from the rest of the fosmid after digestion with BamHI and NsiI. The 5kb fragment was then ligated into pBBR1mcs (Kovach, Elzer et al. 1995) that had been digested with BamHI and PstI. The ligation was transformed into JM109 via electroporation and selected on LB chl 25 mg/L plates. The transformants were screened using primers specific for the desired insert. The resulting plasmid, pJ25, was then harvested and transformed into *E. coli* epi300 in order to afford comparisons with the fosmid in the same genetic background.

PCR strand overlap extension (SOE) strategy was employed to co-express *ipfH,* a putative ferredoxin reductase gene, and *ipfI,* a ferredoxin gene, both of which were also located on pFOS3G7. SOE employed PCR primers with additional 5' homology overlaps between the two PCR products that allowed for artifical joining of the two PCR products. An initial round of PCR using a 50C anneal and 1.5 minute extension with a pFOS3G7 template and the SOE primers (Table 3.2) was used. The products, which were of the predicted size, were gel purified and used as template for three cycles of

primer-less PCR under the same conditions. IpfFeDoxFsew and ipfFeDoxRedRsew were then added and PCR performed under the same conditions, yielding a combined product of the predicted size. The SOE product was t-cloned into pGEMt-easy, sequence verified, and transformed into *E. coli* epi300 pJ25.

Table 3.2. Primers used in this study. 1: pGEMt- Easy vector system, Promega Corporation, Madison, WI. 2: EZ::TN <TET-1> Insertion Kit, Epicentre, Madison, WI.

name	sequence	source
ipfAF	TGACATGATTGAAGGTCGCACCGA	this study
ipfAR	ATGATGTATCTCCTTGCGCGTCCT	this study
ipfBF	AGCGTATTGATCACCACCTCATCC	this study
ipfBR	TTTGCAGACGCAAGGGCAACTCTT	this study
ipfDF	AAACATGTGTCGATTACCGCGGAC	this study
ipfDR	ATGAATGCGAGAAATGCCTCGTCG	this study
ipfEF	AATGGTGGCTGCCTTGACTACCA	this study
ipfER	TCGATGCTTTCTTGCGTCAGGATG	this study
ipfFF	TTTCGACCGACAAGGTCTGGTGTT	this study
ipfFR	ACGGCGGTAGCCATCAAACATTTC	this study
T7F	GTAATACGACTCACTATAGGGC	1
M13R	GCAAACAGCTATGACCATG	1
FP1	GGGTGCGCATGATCCTCTAGAGT	2
RP1	TAAATTGCACTGAAATCTAGAAATA	2
ipfFeDoxFsew	TACCGCCGAGCAGGAATATTACAGCCGCGACA	this study
ipfFeDoxRsew2	GAAGCGGGTACATTCCTCCAGACGGTCCTC	this study
ipfFeDoxRedFsew	GGAGGAATGTACCCGCTTCACGCACACAATCTA	this study
ipfFeDoxRedRsew	TTTCACCGCAGGCCTATGCCGC	this study
ipfDlambdaF	CATGGGGACGAGGCTGTTCCCAGTCTGGCATTCATCATCTGACGCGCACCGTGTA GGCTGGAGCTGCTTC	this study
ipfDlambdaR	AATGCCTCGTCGTAGCGGTGAAAAAAGGTGCTATCCATCAATGGCTCCAGATGGG AATTAGCCATGGTCC	this study
ipfAlambdaF	GGAAATACTTTCAGCGCCAGTTTTTACACTATTTTTGTGTAGGCTGGAGCTGCTTC	this study
ipfAlambdaR	CCCGAAGGGGGGCGGATGAGGTGGTGATCAATACGCATGGGAATTAGCCATGGTCC	this study
ipfBlambdaF	AGCGTCCTGCGAGCAGGACGCGCAAGGAGATACATCGTGTAGGCTGGAGCTGCTTC	this study
ipfBlambdaR	TGGTCTTTTTCTTCACTCGCGTGACCATGGTGCGCGATGGGAATTAGCCATGGTCC	this study
ipfElambdaF	AGCCCACACCGCACCCTCGCCTGACTGGAGCCATTGGTGTAGGCTGGAGCTGCTTC	this study
ipfElambdaR	TCTTGCCAACATAACTTGTCTCCTTGGTTTTAAGTTATGGGAATTAGCCATGGTCC	this study

Metabolic analyses of subclones

The metabolic capabilities of *E. coli* epi300 pJ25 pGEM:*ipfHI* (J25HI) were studied by adding substrates to mature LB-grown cultures. J25HI was compared directly

to *E. coli* epi300 pJ25, *E. coli* epi300 pGEM:*ipfHI*, and *E. coli* epi300 with no vector. Initial expression assays utilizing 25ppm chloramphenicol with the addition of substrate at the time of inoculation failed to demonstrate activity. To promote higher copy numbers of the plasmids prior the introduction of substrate, cultures were grown into stationary phase before introduction of substrate. In order to avoid negative selection effects exerted by the putative toxic catcholic metabolites, substrate addition was delayed until 24 hours after inoculation. At 24 hours 1mM of test substrate added as a 1M solution in ethanol was spiked into the cultures. Test substrates were ibuprofen and phenylacetate both of which have been previously shown to be converted to corresponding deacylated catechols by *Sphingomonas* Ibu-2 (Chapter 2). The media consisted of LB and 100ppm chloramphenicol and/or 150ppm ampicillin. Following the addition of test substrate, cultures were incubated at 37C for 18 hours.

For direct visualization of catechols, ferric choride was added to 150ul of culture to a final concentration of 1.5mM in 96-well plate format. Additionally, HPLC was used to directly quantify substrate and catechols. 40:60 methanol:40mM acetic acid running buffer was used for separation of phenylacetate (10.6 minutes) and catechol (4.3 minutes). 70:30 methanol:40mM acetic acid was used to separate ibuprofen (13.2 minutes) and a peak at 3 minutes presumed to be isobutylcatechol due to its high absorbance at 280nm. A detection wavelength of 220nm was used for the aromatic acids while a detection wavelength of 280nm was used for the catechols. Standard curves were used to quantify the analytes. Because no standard exists for isobutylcatechol, the catechol standard curve was used to approximate isobutylcatechol concentration.

Complementation of pFOS3G7 ipfABDEF mutants

pFOS3G7Tn:*ipfF* was complemented by the cloning *ipfF* into pGEMt-easy using the primers ipfFF/ipfFR that had been designed so as to include the native ribosomal binding site (Table 3.2). Attempts to complement other four genes (*ipfABDE*) using the same approach described above were unsuccessful (results not shown). In order to reduce the influence of possible polar effects introduced by the Tn5 cassette, markerless mutants were created using the lambda red system using a protocol modified from Datsenko and Wanner (Datsenko and Wanner 2000). pKD4 insertion cassette primers with 36-50bp overlap homology to target gene-flanking regions (Table 3.2) were used to generate insertion cassettes and transformed into *E. coli* epi300 pFOS3G7 pKD46 with arabinose induction as described previously (Datsenko and Wanner 2000). However, the pCC1 fosmid is induced to high copy number by arabinose which led to difficulty in isolating insertion mutants presumably due to the loss of mutated fosmid during recovery following transformation with the insertion cassette. In order to achieve acceptable recovery of insertion mutants, 10mM arabinose was therefore added during both the recovery and initial plating which increased recovery of insertion mutants by more than an order of magnitude (data not shown). The cassettes were removed by electroporation and induction of pCP20 as previously described (Datsenko and Wanner 2000). Insertions and deletions were confirmed by PCR analysis.

Except in the case of *ipfF,* initial attempts to complement the loss of function knockouts using expression constructs that included the native ribosomal binding site regions were unsuccessful (data not shown). In order to address the possibility that the lack of complementation was due lack of efficient translation, complements were created

using primers with artificial stop codons and ribosomal binding sites added to the 5' end of the forward primers (Figure 3.2). PCR was performed using proof-reading polymerase followed by monoadenylation of the resulting blunt-ended product by adding taq polymerase and ATP following purification. Resulting plasmids were sequenced and given the suffix rbs, then transformed into pFOS3G7 deletion mutants or transposon mutants and screened for catechol accumulation. Because pGEM:*ipfE*rbs failed to complement pFOS3G7Δ*ipfE*, an attempt to complement using pGEM:*ipf*DErbs was undertaken.

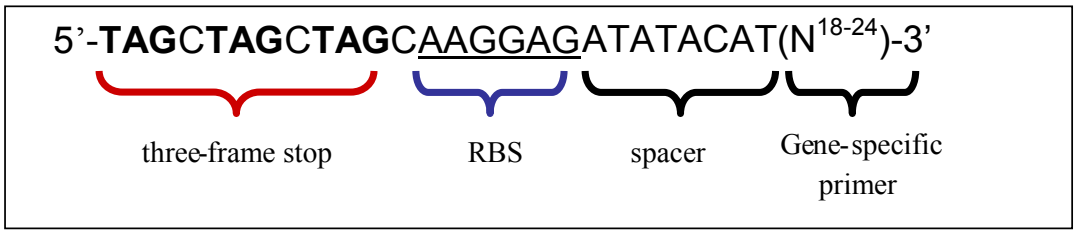

Figure 3.2 expression vector forward primer strategy. RBS; ribosomal binding site

In silico analyses

Fosmid transposon library clone sequences were assembled into contiguous units using SeqMan (DNAStar, Inc., Madison, WI) and searched for open reading frames (ORFs) using GeneQuest (DNAStar, Inc., Madison, WI). Translated ORFs were subjected to Blastp analysis against the Swiss-prot database at the National Center for Biotechnology Institute (NCBI) website (Altschul, Madden et al. 1997). Conserved domain analysis was conducted using the CDD tool at NCBI (Marchler-Bauer, Anderson et al. 2003; Marchler-Bauer and Bryant 2004). Phylogenetic trees were built using the COBALT multiple alignment tool (Papadopoulos and Agarwala 2007) using Fast

Minimum Evolution tree building method with Grishin distance. Three-dimensional structure modeling was performed using Swiss-model and visualized using Swiss-PDB viewer (Peitsch 1995; Arnold, Bordoli et al. 2006; Kiefer, Arnold et al. 2009). Protein amino acid sequence alignments were performed using MegAlign (DNAStar, Inc., Madison, WI).

Expression of ipfD *and* ipfE *in* E. coli *K12* paa *operon mutants*

In order to investigate the interaction between *E.coli* K-12 phenylacetate metabolites (Chapter One, Figure 1.8) and IpfDE, the *ipfDE* pGEMt-easy expression vector described above was electroporated into *E.coli* K-12 Δ*paaG*, Δ*paaZ*, and Δ*paaJ* mutants and screened for effects on growth rate, catechol generation, and for effects on the rate of phenylacetate metabolism and 2-hydroxyphenylacetate accumulation. The growth medium was 0.2% glycerol with 7mM phenylacetate, 1mM IPTG, and 150ppm ampicillin adjusted to pH 7. Strains with pGEM:*ipfDE*rbs were tested against control strains harboring empty pGEMt-easy vector. Phenylaceteate and 2-hydroxyphenylacetate were assayed via HPLC using the same equipment described above with a 40:60 methanol:40mM acetic acid eluent. LB medium with 1.5 mM ferric chloride and 100ppm p-toluidine along with 1mM IPTG and 150 ppm ampicillin was employed for the detection of catechol.

Metabolism of Phenylacetyl Coenzyme A in cell-free extracts

Further metabolism of phenylacetyl CoA was measured in reactions containing 50 µl 2mM phenylacetyl CoA (Sigma, St. Louis, MO), 50 µl 2mM NADH, and 100 µl cell-

free extract. Crude cell-free extracts of *E. coli* clones were prepared by centrifuging 100ml of mid-exponential growth phase 1mM IPTG or 10mM arabinose induced culture, washing, and resuspending in a minimal volume of sonication buffer (40 mM potassium phosphate, 20% glycerol, 1mM PMSF, 1mM DTT, pH 7.4). Approximately 0.1g of 0.1 mm glass beads was then added and the pellets were bead-beaten for three minutes (MiniBeadbeater-8, Biospec Products, Bartlesville, OK). The pellets were then spun down as described above. In both cases, the protein content of the supernatant containing the crude extract was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The reactions were left at room temperature for one hour, whereupon the reaction was terminated by the addition of 20 ul 4M trichloroacetic acid. In order to quantify phenylacetyl CoA, the samples were analyzed via HPLC with UV-Vis detection as described above.

GC/MS was used to test for the appearance of putative metabolites of phenylacetyl-CoA. After acidification of the samples to terminate the reaction, the pH was raised to 10 by the addition of sodium hydroxide in order to promote alkaline hydrolysis of the coenzyme group (Mangino, Zografakis et al. 1992). The effectiveness of this procedure was confirmed by noting the disappearance of phenylacetyl-CoA by HPLC in a separate assay. After 20 minutes, aqueous acetylation was performed by previously described methods (Murdoch and Hay 2005) in order to selectively conjugate aromatic hydroxyl groups. The sample was then acidified to pH 2.5 and precipitates were removed via centrifugation. The sample was then extracted with ethyl acetate and dried over a sodium sulfate column. The ethyl acetate extracts were methylated using diazomethane and dried once more over a sodium sulfate column. GC/MS was

performed using the methods described above. A standard of 2-hydroxyacetic acid, a hypothetical metabolic byproduct, was also subjected to the same treatment and analysis.

Results

Ibu-2 fosmid library

E. coli epi300 pFOS3G7 was the only clone of the 900 clone Ibu-2 fosmid library that accumulated visible brown pigment when exposed to ibuprofen in liquid media. The presence of isobutylcatechol (Murdoch and Hay 2005) in ethyl acetate extracts of acetylated culture supernatant from *E. coli* epi300 pFOS3G7 grown in LB and exposed to 500 mg/L ibuprofen was confirmed via GC/MS (retention time 15.7 minutes with major peaks (relative abundance) of 123(99), 166(100), 208(17), 250 (4)) (Figure 3.3).

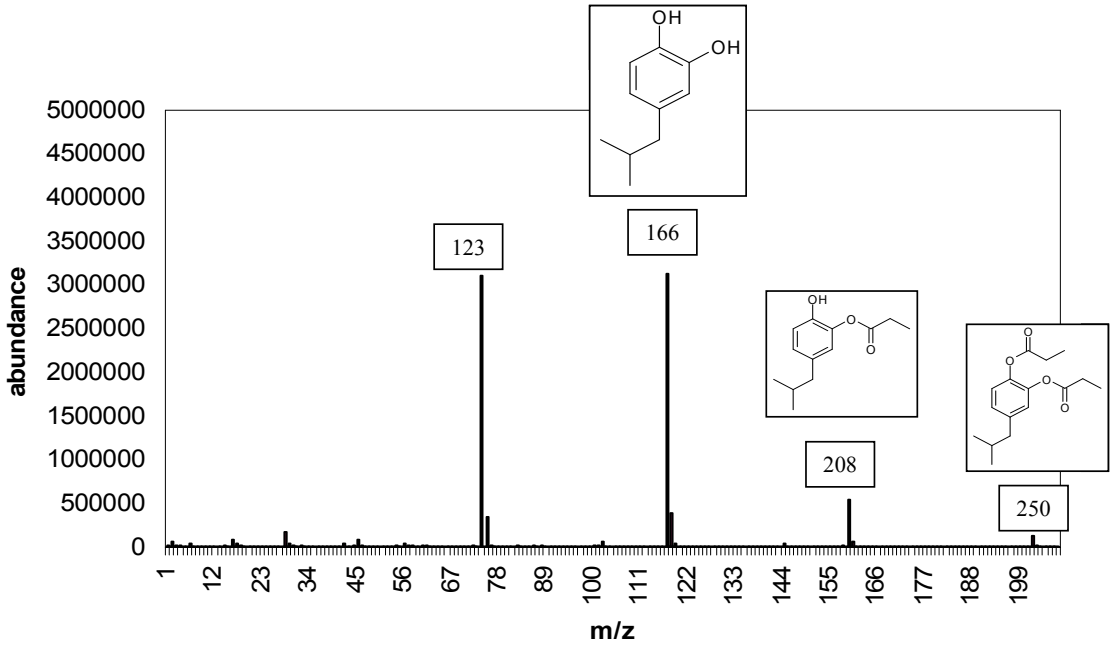

Figure 3.3. Mass-spectrum of acetylated isobutylcatechol detected in extracts of *E. coli* epi300 pFOS3G7 supernatant.

Screening and Characterization of E. coli epi300 pFOS3G7 transposon library

When the 96-clone transposon library of *E. coli* epi300 pFOS3G7 was screened for catechol accumulation in the presence of ibuprofen, seventeen clones no longer accumulated the characteristic dark catecholic polymerization product, indicating a loss-of-function. Sequencing of the DNA surrounding the transposon insertion and analysis of the sequence information revealed that thirteen of the *E. coli* epi300 pFOS3G7 transposon insertion clones harbored a transposon in a 5kb region of Ibu-2 DNA, while the other four clones had the transposon in the vector. Sequence analysis (DNAStar, Inc., Madison, WI) revealed that this 5kb region contained five open reading frames (ORFs) (Figure 3.4) with sequence similarities to aromatic and other metabolic enzyme-encoding genes described in more detail below. All five ORFs had representative knockouts

amongst the loss-of-function mutants. Only *ipfB* and *ipfF* had recognizable conserved ribosomal binding sites (Table 3.3).

In addition to *ipfABDEF,* two open reading frames with sequence similarities to a ferredoxin reductase (*ipfH*) and a ferredoxin (*ipfI*) were identified (Figure 3.4). *ipfH* was identified by observing that a particular transposon library mutant (pFOS3G7Tn:*ipfH*) accumulated dark catecholic coloring at a slightly slower rate. *ipfI* was fortuitously identified during random sequencing of pFOS3G7 transposon library clones.

Table 3.3. DNA immediately upstream of *ipf* open reading frames. Purine-rich regions with homology to conserved ribosomal binding site are underlined. **Only *ipfB and ipfF* had strong RBSs.**

ipfA
CAGCGCCAGTTTTTACACTATTTTTstart

ipfB
AGCAGGACGCGCAAGGAGATACATCstart

ipfD
TGGCATTCATCATCTGACGCGCACCstart

ipfE
CACCCTCGCCTGACTGGAGCCATTGstart

ipfF
AAACTTAAAACCAAGGAGACAAGTTstart

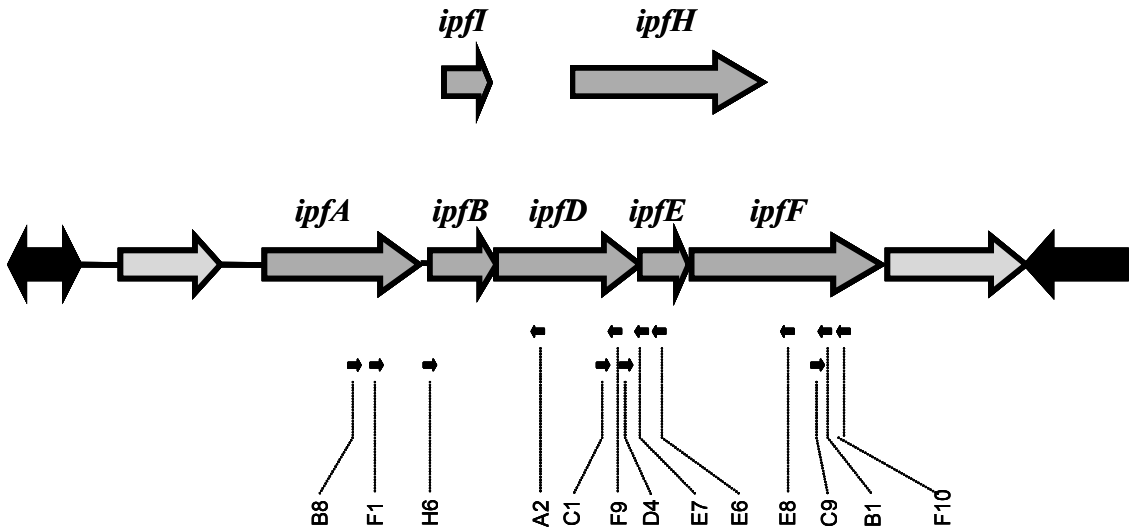

Figure 3.4. Open reading frames found on pFOS3G7. Large black arrows represent ORFs with high similarity to conserved transposase genes. Small black arrows represent the location and orientation of loss-of-function transposon insertions. ORFs described in this study are named.

Metabolic analyses of clones and constructs

Deletions of genes following application of the lambda-red system was successful as indicated by PCR analysis (Figure 3.5). Ibuprofen disappearance assays revealed several trends. First of all, they clearly demonstrated that expression of pFOS3G7 in *E. coli* epi300 caused the disappearance of ibuprofen from the culture supernatant (Figure 3.6). *E. coli* epi300 pFOS3G7 loss-of-function mutants representing each putative metabolic gene caused partial or complete loss of ibuprofen disappearance (Figure 3.6) while not producing visible catecholic polymers (Figure 3.7) or any isobutylcatechol detectable by GC/MS (data not shown). Notably, pFOS3G7Tn:*ipfF* did not degrade ibuprofen at all.

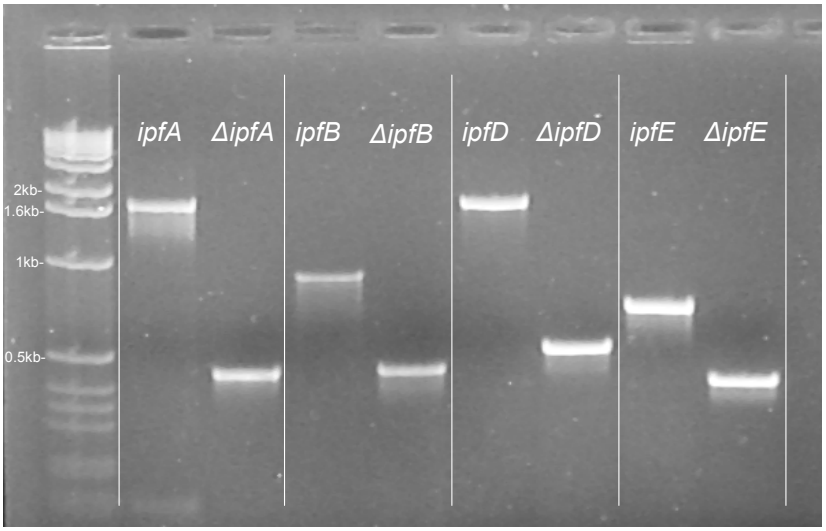

Figure 3.5. PCR of indicated *ipf* genes using either intact pFOS3G7 or makerless mutants. All products were of the predicted size for targeted gene deletion.

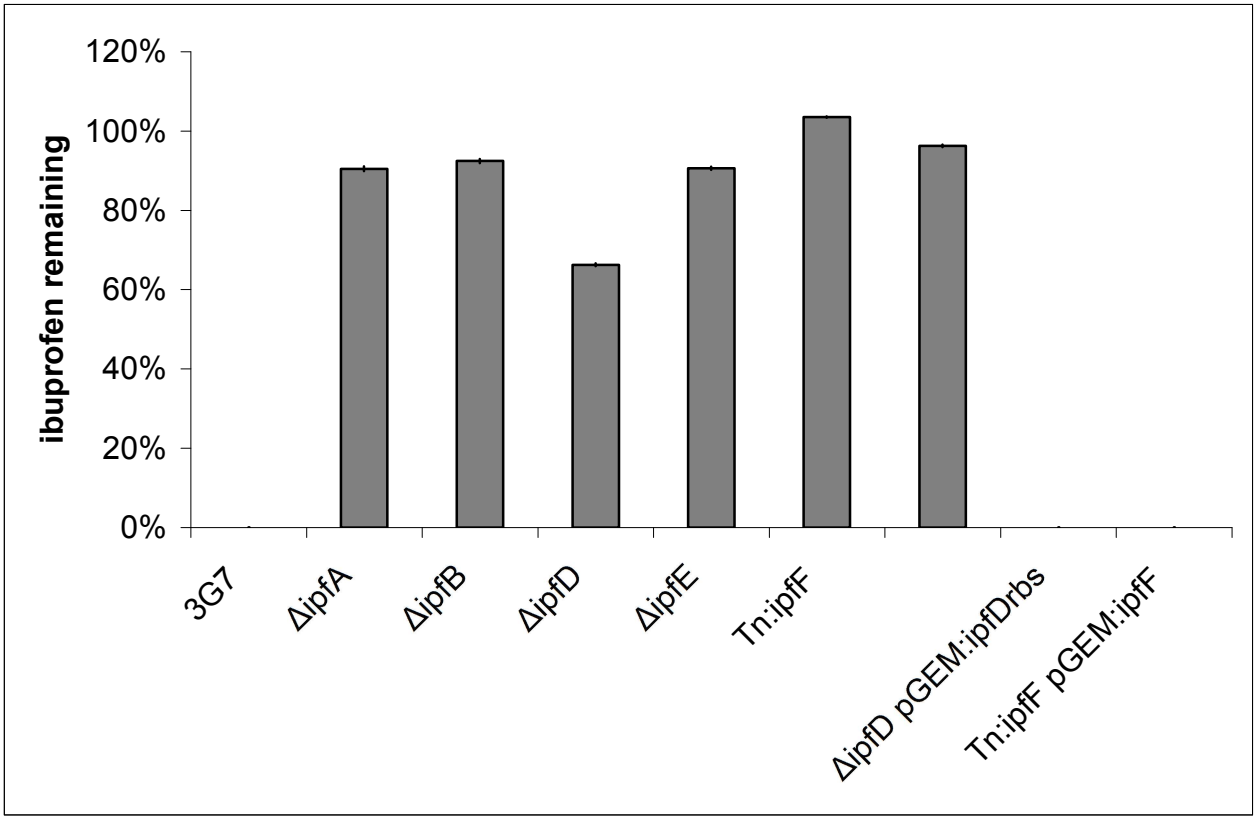

Figure 3.6. Percentage of 50ppm ibuprofen remaining after two days of incubation in *E. coli* epi300 pFOS3G7, loss-of-function mutants, and the two successful complementation

constructs as determined by HPLC analysis of the cultures shown in Figure 3.17. n = 3, standard deviations were too small to be visualized effectively.

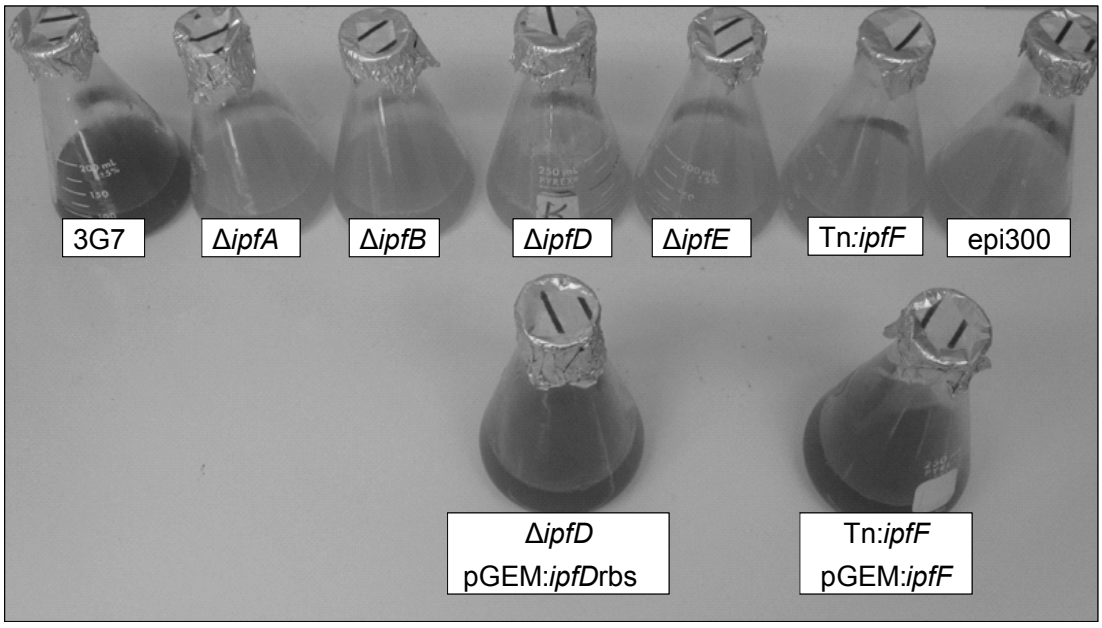

Figure 3.7. Catecholic polymer accumulation in *E. coli* epi300 pFOS3G7, loss-of-function mutants, and the two successful complement constructs grown in LB broth with 50 ppm ibuprofen and 1.5mM ferric chloride.

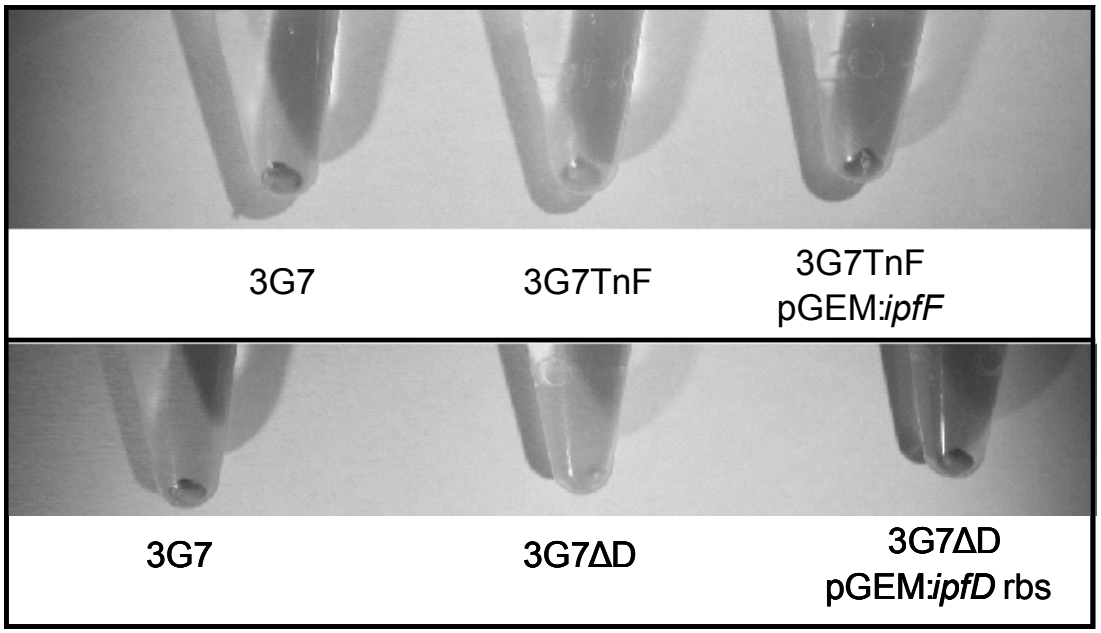

Figure 3.8. Centrifuged knockout and complement strains showing the tendency of catecholic polymers created in the presence of 1.5mM ferric chloride to pellet along with the cell mass.

Markerless deletion mutants of the individual *ipf* genes (*ΔipfA, ΔipfB, Δ ipfD,* and *ΔipfE*) also failed to produce catecholic metabolites (Figures 3.7 and 3.8) and eliminated less ibuprofen than intact pFOS3G7 (Figure 3.6). Complementation of pFOS3G7*ΔipfD* with pGEM:*ipfD*rbs and pFOS3G7Tn:*ipfF* with pGEM:*ipfF* restored catechol generation and ibuprofen disappearance (3.6-3.8), although we were unable to complement the other mutants.

Results of HPLC analysis of *E. coli* epi300 pFOS3G7Tn:*ipfH* were consistent with the observation that dark catecholic metabolites accumulate more slowly in this clone (Figure 3.9).

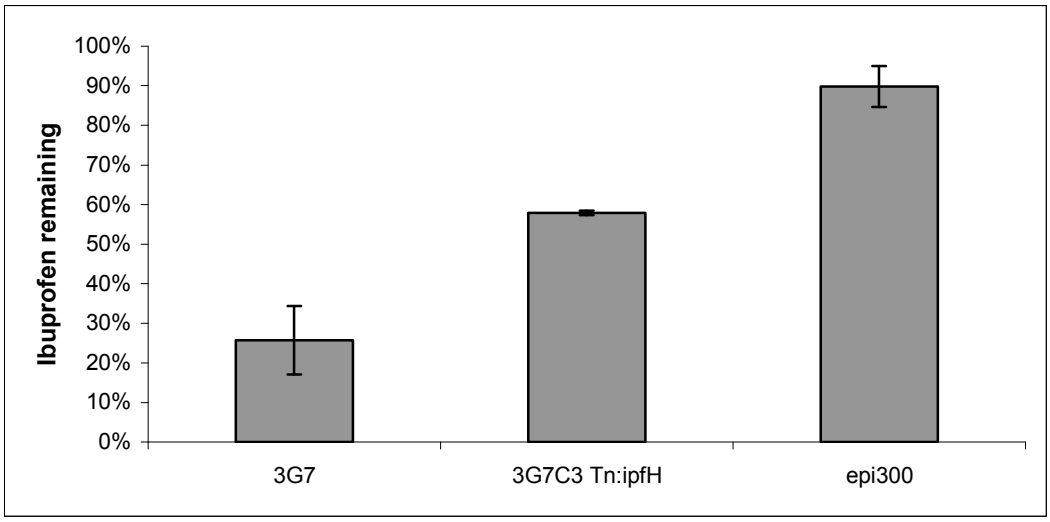

Figure 3.9. % of 50ppm ibuprofen metabolizied by *E. coli* epi300 pFOS3G7, *E. coli* epi300 pFOS3G7Tn:*ipfH,* and epi300 negative control after four days as determined by HPLC analysis.

When *ipfABDEF* were subcloned into pBBR1mcs to create pJ25, *E. coli* harboring this plasmid produced trace amounts of isobutylcatechol when grown with ibuprofen as detected by GC/MS (data not shown), but no polymerization products were visible nor were levels detectable via HPLC analysis. *E. coli* epi300 pJ25 pGEM:*ipfHI*

cultures showed dark brown coloration in response to ferric chloride addition (Figure 3.10) while single vector or vectorless controls contained no detectable dark coloration. HPLC analysis revealed that *E. coli* epi300 pJ25 pGEM:*ipfHI* degraded at least 50% more of the ibuprofen and phenylacetate test substrates than single vector or vectorless controls. Additionally, HPLC analysis detected catechol in phenylacetate only in dual-vector cultures. The disappearance of phenylacetate in the dual vector culture (0.58 mM) corresponded closely with the accumulation of catechol (0.37 mM) (Figure 3.11). The putative isobutylcatechol peak was quantified using the catechol standard curve because no standard for isobutylcatechol is available. 0.42 mM ibuprofen disappeared from the pJ25 pGEM:*ipfHI* culture and approximately 0.25 mM isobutylcatechol appeared (Figure 3.12).

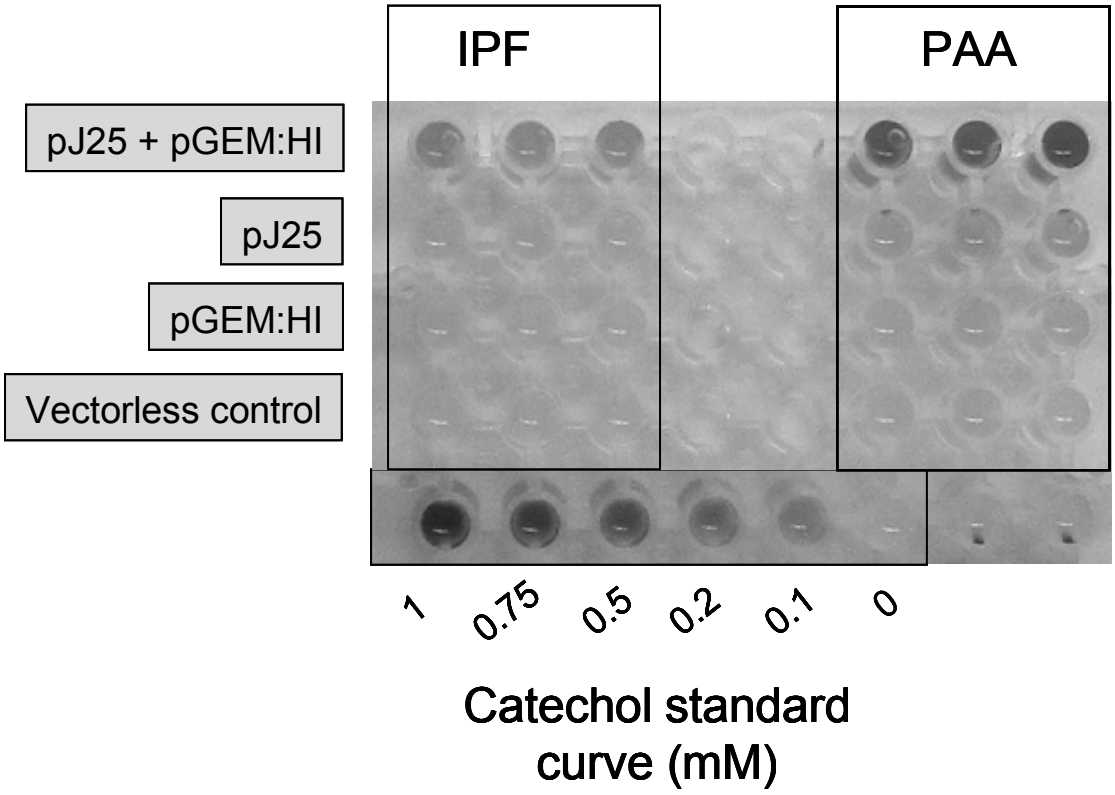

Figure 3.10. 150ul of *E. coli* epi300 harboring the indicated plasmids following 18 hours of incubation with 1mM ibuprofen (IPF) or phenylacetate (PAA) with 1.5 mM ferric chloride for catecholic metabolite visualization.

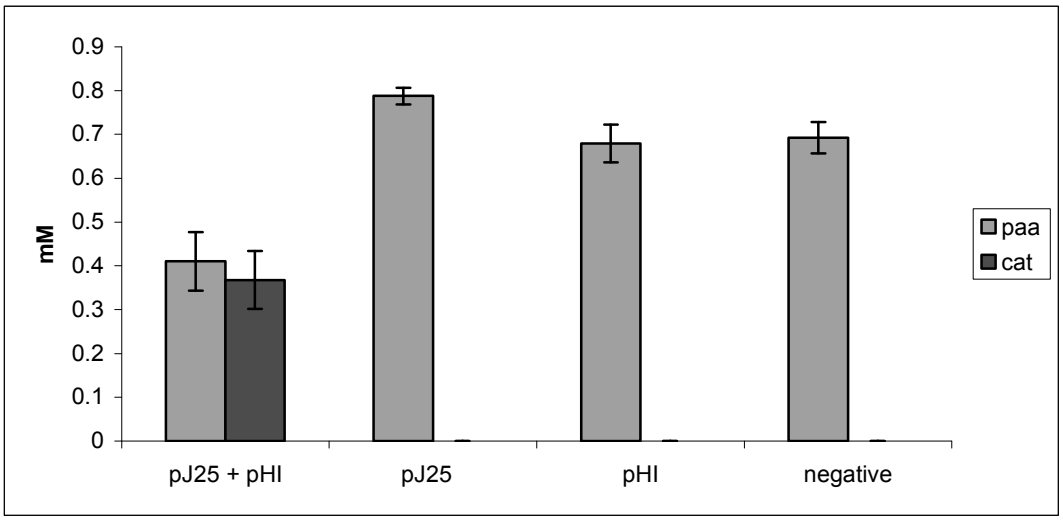

Figure 3.11. Phenylacetate and catechol concentration in *E coli* epi300 cultures harboring pJ25 and/or pGEM:*ipfHI* (pHI) following 18 hours of incubation 1 mM phenylacetate.

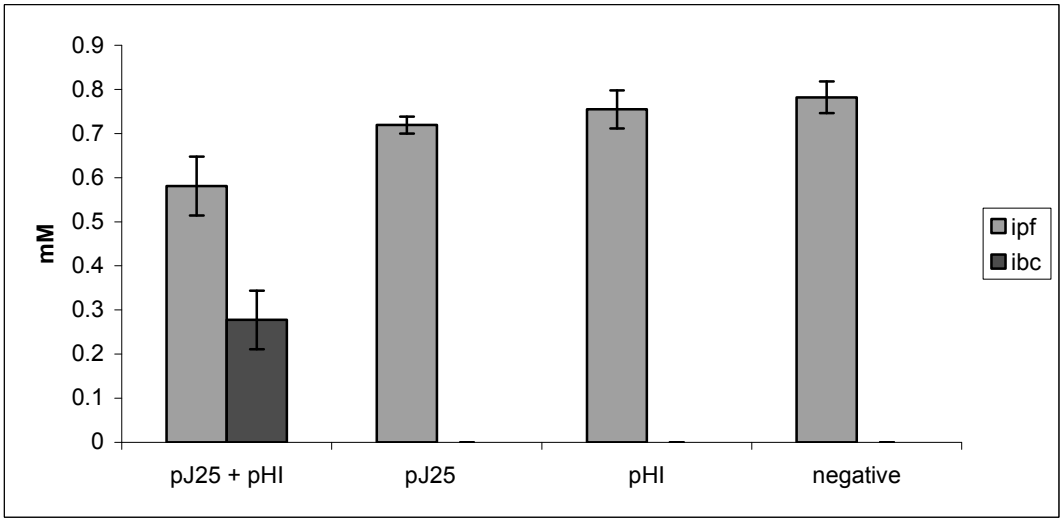

Figure 3.12. Ibuprofen and isobutylcatechol concentration in *E coli* epi300 cultures harboring pJ25 and/or pGEM:*ipfHI* (pHI) following 18 hours of incubation 1 mM ibuprofen.

Phenylacetyl coenzyme A disappearance in E. coli pGEM:ipfAB cell-free extract

The rate of phenylacetyl coenzyme A disappearance in both *E. coli* pFOS3G7 (172 nmol/(mg protein*hr)) and JM109 pGEM:*ipfAB* (161 nmol/(mg protein*hr)) cell-

free extracts were significantly higher than in cell-free extract of JM109 (123 nmol/(mg protein*hr)) ($p<0.05$) (Figure 3.13). While no novel peaks were detected via GC/MS analysis, the peak area of phenylacetic acid was approximately 90% less in the JM109 pGEM:*ipfAB* sample than it was in the JM109 control.

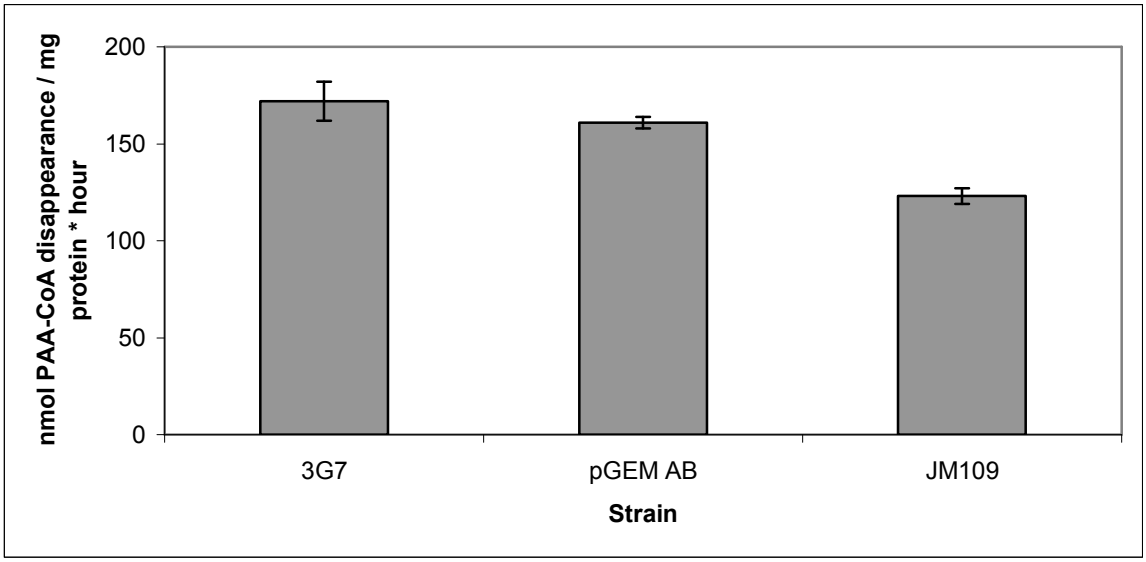

Figure 3.13. Disappearance of phenylacetyl CoA (PAA-CoA) in cell-free extract experiments. PAA-CoA was quantified via HPLC. n=3. All data points are significantly different from one another as calculated by student's t-test ($p<.05$).

Expression of ipfDE in E. coli K12 paa *mutants*

In order to gain a greater understanding of the role of IpfD and IpfE, we expressed them in *E.coli* K12 mutants that had lost the ability to cleave the ring of phenylacetic acid (*paaG, paaZ,* and *paaJ* mutants). The genes were co-expressed due to the co-dependency of bacterial SCPx thiolases and DUF35 proteins that has been previously observed in the Phl (Bangera and Thomashow 1999) and Bbs (Leuthner and Heider 2000; Kube, Heider et al. 2004; Kuhner, Wohlbrand et al. 2005) pathways. This work was undertaken prior to the recent publication by Teufel *et al* (Teufel, Mascaraque et al. 2010) with the

hypothesis that Ibu-2 and K12 might share a common hydroxylated, CoA-ligated intermediate which could be acted upon by IpfD and IpfE, resulting in isobutylcatechol production. While no isobutylcaechol was detected, expression of *ipfDE* caused a 57% increase in the amount of PAA disappearance ($p<0.05$) and a 67% increase ($p<0.05$) in the amount of 2-hydroxyphenylacetate detected in the supernatant of the *E. coli* K12 Δ*paaG* mutant, but not in the wild type or either of the other two mutants implicated in ring fission (Figure 3.14, also see Figure 1.8 for the *paa* pathway). The 2-hydroxyphenylacetate accounted for the majority of metabolized phenylacetate in strain K12 Δ*paaG.*

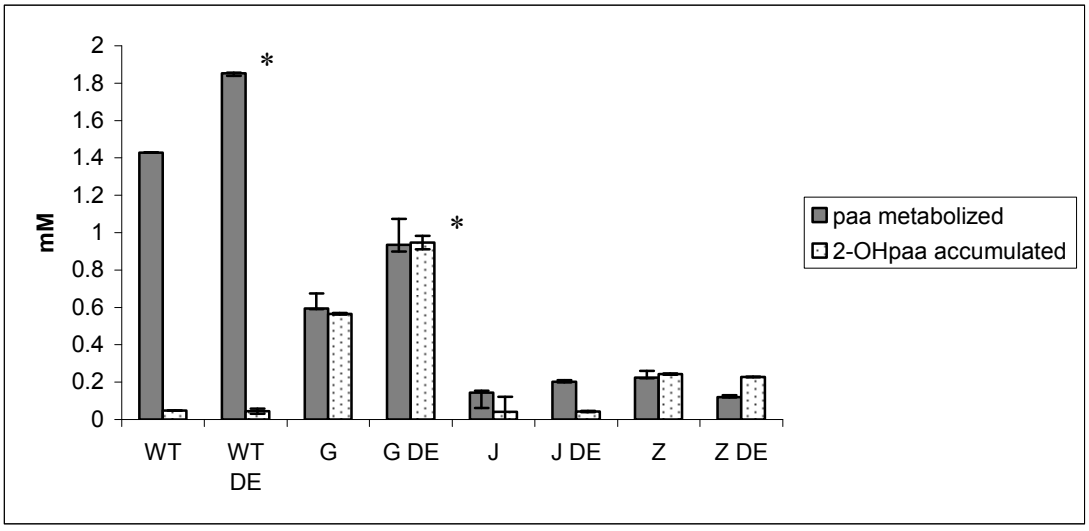

Figure 3.14. A; Concentration of phenylacetate remaining after three days in *E. coli* K12 cultures (7mM starting concentration). The strains are WT, wild type; G, Δ*paaG*, J, Δ*paaJ*; Z, Δ*paaZ.* "DE" indicates presence of pGEM:*ipfDE*rbs. All strains without pGEM:*ipfDE*rbs harbored the empty pGEMt-easy vector. All strains were grown in 0.2% glycerol MSM with 1mM IPTG induction at 37C. Asterics indicate significantly higher 2-hydroxyphenylacetate ($p<0.05$)

Discussion

Previously (Murdoch and Hay 2005), isobutylcatechol was detected in ibuprofen-grown *Sphingomonas* Ibu-2 cultures when and only when catechol-meta-cleavage activity had been poisoned by the addition of 3-fluorocatechol. The removal of the acid side-chain and consequent creation of the corresponding catechol was demonstrated for a variety of phenylacetic acids by ibuprofen-induced cultures (Figure 3.1). GC/MS spectra corresponding to isobutylcatechol meta-cleavage products were also observed (Table 2.1).

In order to further study this pathway, a fosmid library was constructed in *E. coli* using *Sphingomonas* Ibu-2 chromosomal DNA. *E. coli* epi300 pFOS3G7 turned brown in the presence of ibuprofen, indicative of accumulation of a catecholic metabolite. The disappearance of ibuprofen from *E. coli* epi300 pFOS3G7 and accumulation of isobutylcatechol was confirmed via HPLC and GC/MS respectively. Creation and screening of a transposon library of pFOS3G7 led to the discovery of five genes (*ipfABDEF)* that, when knocked out, prevented isobutylcatehcol accumulation and slowed the disappearance of ibuprofen.

ipfA

Sequence analysis of *ipfA* revealed that its predicted protein product contained two conserved domains an N-terminal Rieske domain and a C-terminal catalytic domain similar to those of the alpha subunit of anthranilate 1,2-dioxygenase (AntDO, CD03538) conserved domain and related Rieske-type non-heme iron aromatic ring-hydroxylating

oxygenase (RHO, CD00680) conserved domain. The general RHO domain is typified by having a large hydrophobic pocket that affords aromatic substrate specificity while proteins containing the AntDO subfamily domain are specific for aromatic acids (Gibson and Parales 2000; Ferraro, Gakhar et al. 2005). The multi-domain architecture is similar to those of 1,2-benzoate dioxygenase, 1,2-anthranilate dioxygenase, and 3-phenylpropionate dioxygenases. There was no detectable similarity at any level to the monooxygenase encoding genes of the *E. coli paa* operon *paaABCDE.* Thus, phylogenetic analysis clearly places IpfA in the of the RHO superfamily (Table 3.4).

Table 3.4. The substrates, similarity measures, and confidence levels of comparisons between the predicted amino acid sequence of *ipfA* and characterized large subunits of dioxygenases found in the Swiss-prot database.

Accession	Description (alpha subunit of) Organism	% identity	% positive similarity	Query coverage	E value
ABY62759.1	indole 3-acetic acid dioxygenase IacC *P. putida*	34	51	94%	1.00E-62
P37333.3	biphenyl 2,3-dioxygenase *Burkholderia xenovorans*	29	45	93%	6.00E-45
AAB62285.1	p-cumate 2,3-dioxygenase *P. putida*	29	43	92%	2.00E-44
BAB78521.1	2,4-dichlorophenoxyacetic acid dioxygenase *Bradyrhizobium sp. HW13*	27	44	84%	4.00E-44
Q7N4W0.1	3-phenylpropionate dioxygenase *Photorhabdus luminescens*	28	46	95%	8.00E-44
Q52028.1	biphenyl 2,3-dioxygenase *P. pseudoalcaligenes*	28	45	93%	1.00E-43
Q07944.1	benzene 1,2-dioxygenase *P. putida*	29	45	92%	2.00E-43
Q51601.3	2-halo benzoate 1,2-dioxygenase *Burkholderia cepacia*	29	46	83%	2.00E-43
A5W4F2.1	benzene 1,2-dioxygenase *P. putida F1*	28	45	92%	3.00E-43
A8A344.1	3-phenylpropionate dioxygenase *Escerichia coli HS*	28	46	94%	3.00E-43
Q83K39.1	3-phenylpropionate dioxygenase *Shigella flexneri*	23	46	94%	4.00E-43
Q52438.1	biphenyl 2,3-dioxygenase *P. sp. KKS102*	28	44	96%	9.00E-43
P07769.2	benzoate 1,2-dioxygenase *Acinetobacter sp. ADP1*	29	47	78%	2.00E-42
ZP06690405.1	anthranilate 1,2-dioxgyenase *Acinetobacter sp. SH024*	28	45	83%	2.00E-41
Q51494.1	napthalene 1,2-dioxygenase *P. aeruginosa*	28	47	94%	3.00E-41
BAH86807.1	2,4-dichlorophenoxyacetic acid dioxygenase *Sphingomonas sp. 58-1*	27	45	81%	3.00E-41
P0A110.1	napthalene 1,2-dioxygenase *P. putida*	29	46	93%	4.00E-41
P23099.1	toluate 1,2-dioxygenase *P. putida*	29	47	70%	1.00E-40
Q46372.1	biphenyl 2,3-dioxygenase *Comomonas testosteroni*	27	44	95%	7.00E-40
O07824.1	napthalene 1,2-dioxygenase *P. fluorescens*	28	45	93%	2.00E-39

The predicted protein product of *ipfA* was most closely related to *iacC* from *P. putida*, though the relationship was still distant (34% identity and 51% similar residues over 94% of its length). *iacC* is predicted to encode the large subunit of an indole-3-acetic acid

dioxygenase (Leveau and Lindow 2005; Leveau and Gerards 2008), though the specifics of the metabolic pathway have not been described. A sequence analysis of available *iac*-region from *P. putida* revealed no genes bearing any similarity to *ipfD, ipfE,* or *ipfF*,

ipfB

Sequence analysis of ipfB reveals strong homology to the beta or small subunits of aromatic dioxygenases. The function of beta-subunits of RHO heterodimers is not well known, but they are believed to serve a largely structural, non-catalytic role (Parales and Resnick 2006). BLAST-P similarity analysis to a variety of dioxygenase beta subunits show 20%+ identity and 40%+ similiarity (Table 3.5).

Table 3.5. The substrates, similarity measures, and confidence levels of comparisons between the predicted amino acid sequence of *ipfB* and characterized small subunits of dioxygenases found in the Swiss-prot database.

Accession	Description (beta subunit of) Organism	% Identity	% Positive similarity	Query coverage	E-value
42151	p-cumate 2,3 dioxygenase *P. putida*	33	47	84%	1.00E-16
P37334.3	biphenyl 2,3 dioxygenase *Burholderia xenovorans*	31	50	85%	4.00E-15
Q52439.1	biphenyl 2,3 dioxygenase *P. sp. KKS102*	32	46	79%	7.00E-14
Q46373.1	biphenyl 2,3 dioxygenase *Comamonas testosteroni*	33	46	83%	1.00E-13
A5W4F1.1	toluene 2,3 dioxygenase *P. putida F1*	25	48	86%	6.00E-13
Q07945.1	benzene 1,2-dioxygenase *P. putida*	25	48	83%	8.00E-13
Q7N4V9.1	3-phenylpropionate dioxygenase *Photorhabdus luminescens*	29	49	87%	1.00E-12
P23100.1	toluate 1,2-dioxygenase *P. putida*	30	50	58%	5.00E-12
Q31XV1.1	3-phenylpropionate dioxygenase *Shigella boydii*	28	48	87%	1.00E-11
Q8XA73.1	3-phenylpropionate dioxygenase *E. coli*	28	47	87%	1.00E-11
P07770.1	benzoate 1,2-dioxygenase *Acinetobacter sp. ADP1*	26	49	53%	2.00E-09
BAB78522.1	2,4-dichlorophenoxyacetic acid dioxygenase *Bradyrhizobium sp. HW13*	26	44	83%	2.00E-08
ABY62760.1	indole-3-acetic acid dioxygenase *P. putida*	23	45	91%	3.00E-08
BAH86808.1	2,4-dichlorophenoxyacetic acid dioxygenase *Sphingomonas sp. 58-1*	24	43	83%	8.00E-07
ADG20790.1	anthranilate 1,2-dioxygenase *Burkholderia sp. CCGE1002*	25	44	74%	9.00E-07
1NDO_F	napthalene dioxygenase *Pseudomonas*	23	45	73%	3.00E-06

ipfD

Analysis of the predicted amino acid sequence of *ipfD* (Marchler-Bauer, Anderson et al. 2003; Marchler-Bauer and Bryant 2004), revealed the presence of a highly conserved domain similar to those found within the condensing enzyme family of proteins related to sterol carrier protein X (SCPx) (E-value = 1.02×10^{-51}) (Stolowich, Petrescu et al. 2002). Condensing enzymes generally catalyze the condensation and/or thiolytic cleavage of acetyl groups to or from beta-keto acids.

There are two main types of condensing enzymes; decarboxylating and non-decarboxylating. Decarboxylating enzymes, which perform Claisen-like, CO_2-evolving condensations, are largely represented by the many variants of polyketide synthesis proteins found within bacteria which are integrally involved in the synthesis of polyketide polymers (Ferrer, Jez et al. 1999; Das and Khosla 2009; Crawford and Townsend 2010).

Non-decarboxylating enzymes, also called beta-keto thiolases are separated into two main groups by sequence phylogeny; thiolases and SCPx thiolases (Heath and Rock 2002). The general thiolase group is typified by the 3-keto thiolases involved in fatty acid synthesis and catabolism such as FadA (Yang, He Yang et al. 1991). ”SCP”-x thiolases are termed “sterol carrier proteins” due to early observations that disruption of the gene led to steroid metabolism disfunction in mammals (Gallegos, Atshaves et al. 2001; Stolowich, Petrescu et al. 2002). While eukaryotic SCPx was originally suspected to be involved in transport of cholesterols and fatty acids (Puglielli, Rigotti et al. 1995; Gallegos, Atshaves et al. 2001), more detailed molecular analyses have demonstrated it’s involvement in two specific metabolic reactions in eukaryotes; bile-acid synthesis (Takeuchi, Chen et al. 2004)(Figure 3.15a) and the beta-oxidation of pristanoyl-CoA, a branched-chain fatty acid (Figure 3.15b) (Wanders, Denis et al. 1997; Verhoeven and Jakobs 2001; Westin, Hunt et al. 2007),.

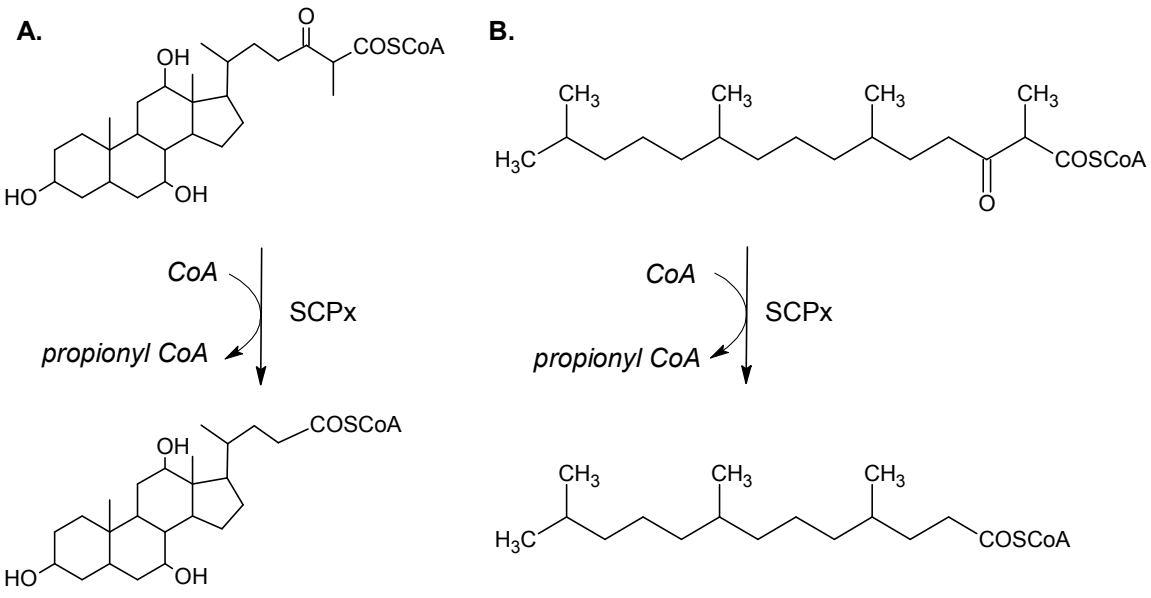

Figure 3.15. Reactions catalyzed by sterol carrier protein X (SCPx) in animals. A; the thiolytic depropionation of 24-oxo-3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA (Takeuchi, Chen et al. 2004): B; the thiolytic depropionation of 3-ketopristanoyl-CoA (Westin, Hunt et al. 2007).

In both cases, SCPx is specifically involved in the beta-oxidation of an alpha-methyl beta-keto fatty acid. While there are over two thousand SCPx-type bacterial genes in the NCBI database, only four have been characterized to any degree. Phylogenetic analysis of IpfD places it within the somewhat divergent bacterial SCPx group which, however, is more closely related to eukaryotic SCPx proteins than it is to other bacterial FadA-type traditional thiolases (Figure 3.16 and Table 3.6).

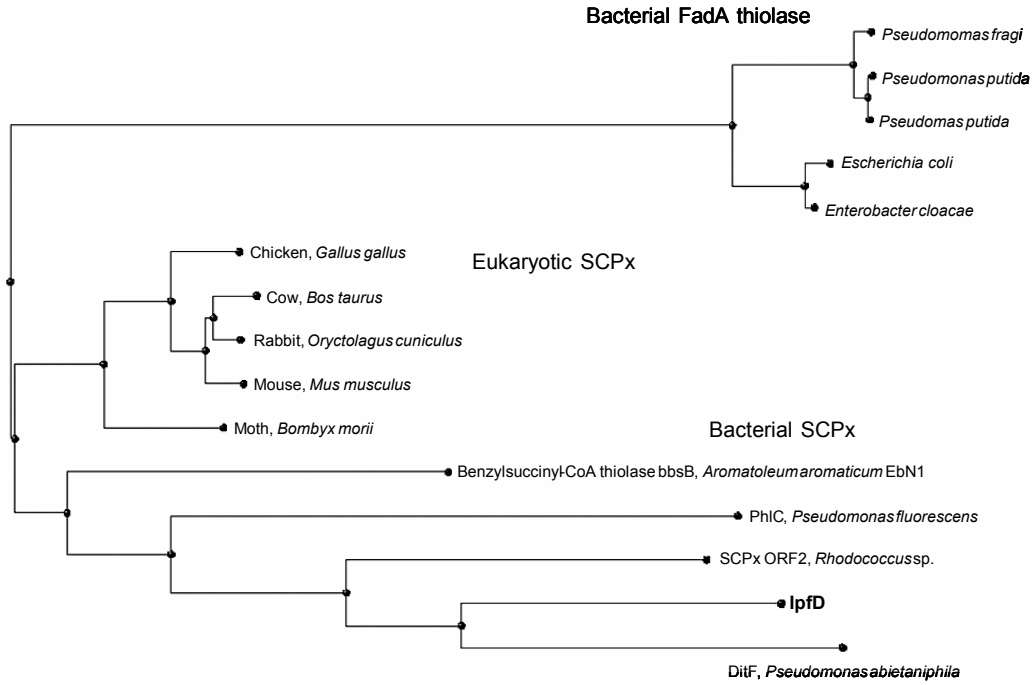

Figure 3.16. Phylogenetic tree of characterized thiolase-type proteins found in the Swiss-prot database and listed in Table 3.5.

Table 3.6. The similarity measures and confidence levels of comparisons between the predicted amino acid product of *ipfD* and and characterized thiolase-type proteins found in the Swiss-prot database.

Accession	Description	Organism	% Identity	% Positive	Query coverage	E-value
AAD21068.1	DitF	*P. abietaniphila*	34	45	89%	1.00E-42
AAK50624.1	Camphor ORF2	*Rhodococcus sp*	33	49	91%	1.00E-37
YP_158078.1	BbsB	*Aromatoleum aromaticum* EbN1	34	50	53%	5.00E-26
Q07598.1	SCPx	*Gallus gallus*	30	44	64%	8.00E-23
NP_001075504.1	SCPx	*Oryctolagus cuniculus*	28	44	68%	1.00E-22
P07857.2	SCPx	*Bos taurus*	27	42	68%	3.00E-21
P32020.3	SCPx	*Mus musculus*	26	43	63%	4.00E-20
NP_001037378.1	SCPx	*Bombyx morii*	28	43	53%	3.00E-17
AAB48108.1	PhlC	*P. putida*	36	52	39%	3.00E-14
P28790.2	FadA	*P. fragi*	30	46	49%	2.00E-06
P21151.3	FadA	*E. coli*	27	40	71%	3.00E-06
Q9R9W0.1	FadA	*P. putida*	30	46	49%	6.00E-06
Q93Q11.1	FadA	*P. putida*	30	46	49%	1.00E-05
Q9F0Y6.1	FadA	*Enterobacter cloacae*	23	36	45%	3.00E-05

Four bacterial SCPx genes have been described at some level. *ditF* (*Pseudomonas abietaniphila*) is a part of a catabolic cluster for abietic acid (Martin and W. 1999; Martin and Mohn 2000)(Figure 3.17a). Knocking out the gene leads to complete loss of growth on abietic acid, though neither the gene product nor the reactions have been described further. PhlC (*Pseudomonas putida*) is required for a polyketide synthase-like action in conjunction with an acyl carrier protein and a domain of unknown function 35 protein in the synthesis of a polyketide antibiotic from acetyl-CoA and malonyl-CoA building blocks (Bangera and Thomashow 1999). The Phl proteins are also able to catalyze the addition of an acyl group directy to an aromatic ring (Figure 3.17b). *Rhodococcus* sp. NCIMB 9784 camphor catabolic cluster ORF-2 (Grogan, Roberts et al. 2001; Roberts, Grodan et al. 2004) may be involved in the metabolism of camphor to alpha-campholinic acid by *camK*, a p450 hydroxylase, though the pathway again has not been characterized nor has the gene's involvement been confirmed. It has however been noted that the pathway involves a Claisen-type reaction in the thiolytic cleavage reaction between the 1,3-ketone carbons (Figure 3.17c) (Grogan, Roberts et al. 2001). While that work provides a hypothetical role for the involvement of an SCPx thiolase, it is fully possible that ORF2 is involved in another aspect of the poorly-described pathway. BbsB from *Aromatoleum aromaticum* EbN1 (Kube, Heider et al. 2004; Kuhner, Wohlbrand et al. 2005) and *Thauera aromatica* (Leuthner and Heider 2000) are part of a relatively better described pathway for the metabolism of toluene to benzoyl-CoA under anaerobic denitrifying conditions. BbsB in conjunction with BbsA performs the decondensation of an alpha-aliphatic beta-keto coenzyme A adduct in a

manner that mirrors the role of eukaryotic SCPx in the metabolism of bile acids and branched fatty acids (Figure 3.17d).

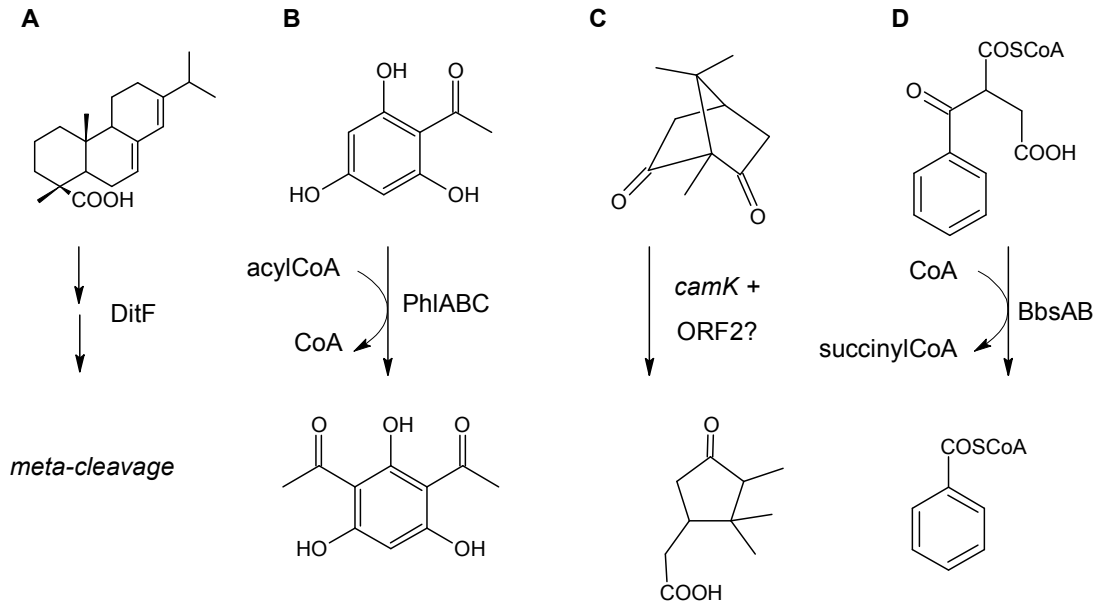

Figure 3.17. General schema of bacterial metabolic pathways that require the action of SCPx thiolases: A; DitF, B; PhlC, C; camphor ORF2, D; BbsB.

Although the branched nature of several of these substrates suggests a similar mechianism may be catalyzed by IpfD, further consideration of ibuprofen and the isobutylcatechol intermediates (Figure 3.1) reveals that a classical thiolase type reaction is not possible due to the fact that there is no way for the beta-carbon, presumably part of the aromatic ring, to be bonded to the sulfur atom of a coenzyme A residue. The thiolase reaction when performed in a reverse catabolic role is defined by the use of the sulfur residue to break the alpha-beta carbon bond; because such a reaction is not apparently possible based on what is known of the *ipf* reaction, no such thiolase reaction can be performed. Both the thiolase and eukaryotic SCPx type proteins share two primary

catalytic residues that have been shown to be crucial to thiolase activity, a cysteine in the 80-100 aa region and a histidine in the 350-400 region (Bangera and Thomashow 1999). Alignment of IpfD and other bacterial SCPx thiolases against well-characterized SPCx and FadA genes shows that IpfD lacks these crucial residues (Figure 3.18); in IpfD, the N-terminal catalytic cysteine is replaced by glycine, a substitution that would be expected to remove catalytic ability. The C-terminal catalytic histidine, a positively charged residue, is replaced by tyrosine, a weakly negatively charged residue, a substitution that is generally associated with changes in catalytic properties (Paganl, Zagato et al. 1994 ; Liu, Moanne-Loccoz et al. 1999). Interestingly, the two bacterial SCPx proteins known to catalyze classical thiolase reactions, PhlC and BbsB, have both of these catalytic residues. More work needs to be done before a mechanistic explanation for the role of IpfD in ibuprofen metabolism can be established, however, given its similarity to SCPx proteins it may interact with the branched sidechain of ibuprofen.

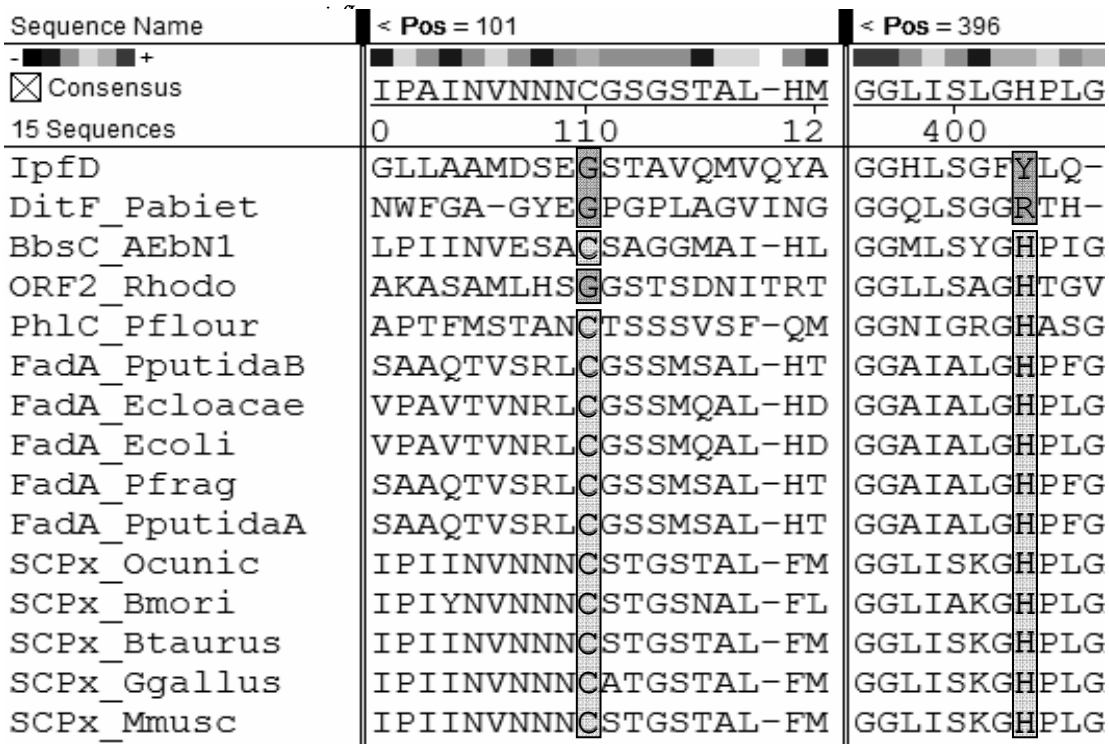

Figure 3.18. Alignment of conserved catalytic residues of IpfD, bacterial and eukaryotic SCPx thiolases, and FadA fatty acid thiolases. Conserved catalytic residues are highlighted in light grey, variations from the conserved catalytic residues are highlighted in dark grey. Created using MegAlign (DNAStar, Inc., Madison, WI).

ipfE

Conserved domain analysis of the predicted amino acid sequence of *ipfF* revealed that it contains a Domain of Unknown Function 35 (DUF35) motif (E-value = 3.71×10^{-11}). Over 600 DUF35 proteins have been found in bacteria and archaea, but none have been identified in eukaryotes. Interestingly, there are 43 cases in the NCBI database when a DUF35 gene is found directly adjacent to nondecarboxylating condensing enzymes, a protein family of which IpfD is predicted to be part. The role played by only two DUF35 proteins have been characterized to any degree. PhlB (*Pseudomonas putida*) plays a role in the polyketide synthase pathway described above. PhlB acts in

conjunction with the SCPx protein PhlC, though its role is unclear. BbsA, which has been characterized in *Thauera aromatica* (Leuthner and Heider 2000) and *Aromatoleum aromaticum* EbN1(Kube, Heider et al. 2004; Kuhner, Wohlbrand et al. 2005) is required for the decondensation reaction catalyzed by BbsB described above (Figure 3.14d) though its role is also unclear. An uncharacterized representative of the DUF35 family from *Sulfolobus solfataricus* was recently crystallized (Krishna, Aravind et al. 2010), revealing a three domain structure (Figure 3.19). At the N-terminal is a conserved but entirely uncharacterized two-helix motif termed the 14-3-3 interacting domain. Next is a zinc-finger motif; zinc-fingers are often associated with nucleotide binding proteins and have been clearly shown to interact directly with nucleotides. Based on this general property and a genome context analysis, it has been hypothesized that in the case of DUF35, this zinc-finger motif interacts with the adenosine moiety of coenzyme A (Krishna, Aravind et al. 2010). At the C-terminal is an oligosaccharide/oligonucleotide binding fold (OB-fold), a double-barreled motif often associated with binding small molecules, which in the case of DUF35 is hypothesized to interact with the hydrophobic end of acyl-CoA molecules based on the presence of hydrophobic amino acid functional groups in the putative binding pocket (Krishna, Aravind et al. 2010). None of the domains is predicted to have any catalytic role.

An alignment and structural modeling of IpfE using the *Sulfolobus* DUF35 template using the Swiss model program (Peitsch 1995; Arnold, Bordoli et al. 2006; Kiefer, Arnold et al. 2009) reveals a strong homology and a stable model (E-value = 1.40e-31) though only for the zinc-finger and OB-fold domains (Figure 3.19). The crystallographers hypothesized that the close proximity of the zinc-finger and OB-fold

motifs, taking into consideration their genomic contexts, suggests that DUF35 interacts specifically with acyl-CoA molecules, perhaps serving a similar role as that played by acyl carrier protein (SCP-2) in eukaryotes. SCP-2 in eukaryotes is tightly associated with SCPx proteins in that it is actually transcribed and translated as a single polypeptide only to be proteolytically cleaved into two proteins following translation. (Gallegos, Atshaves et al. 2001; Takeuchi, Chen et al. 2004; Westin, Hunt et al. 2007). No homology could be detected, however, between SCP2 and DUF35 using BLAST-P.

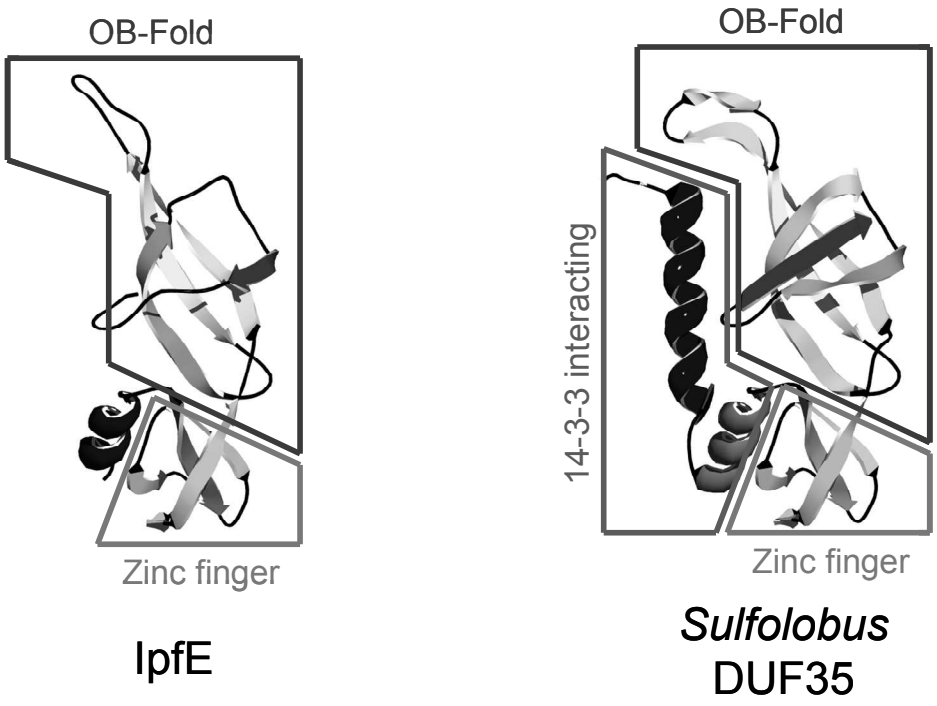

Figure 3.19. Three dimensional model of the predictied amino acid sequence of *ipfE* modelled on the structure of *Sulfolobus* DUF35 (Krishna, Aravind et al. 2010) with conserved domains labeled. Image created using Pov-Ray (Persistence of Vision Raytracer Pty. Ltd., Victoria, Australia).

ipfF

Analysis of *ipfF* is described in Chapter 4. Briefly, the putative gene product of *ipfF* has similarity to coenzyme A ligases of the luxE superfamily.

ipfH

During initial screening of pFOS3G7 transposon mutants, a clone designated pFOS3G7TnC3 that appeared to have stunted accumulation of catecholic polymer was targeted for sequencing analysis. The transposon insertion in clone 3G7C3 was within an ORF that appears to encode for a ferredoxin reductase *ipfH*.

Ibprofen disappearance analysis of *E. coli* epi300 pFOS3G7Tn:*ipfH* via HPLC was in accordance with the observation of lower catechol accumulation (Figure 3.9). TnH metabolized ibuprofen at roughly half the rate as *E. coli* epi300 pFOS3G7. However, the fact that TnH still produces isobutylcatechol from ibuprofen is curious; if IpfH is actually part of an electron transport chain for IpfAB, then it would be expected that knockout out *ipfH* would lead to total loss of function. The fact that the pathway still functions suggests that there is perhaps an additional copy of the gene on pFOS3G7 or that the IpfAB electron transport chain is able to make use of a compatible system in *E. coli.*

Blast-X analysis against the Swissprot protein database revealed strong similarities to a number of bacterial ferredoxin reductase components from aromatic dioxygenase systems (Table 3.7) in addition to several other electron transport chain reductases. This information in combination with the two pieces of metabolic generation support a role for IpfH in the *ipf* pathway as part of the IpfAB electron transport chain.

Table 3.7. Most similar proteins in the Swiss-prot database to IpfH as determined by BlastP.

Accession	Identity	% coverage	E value	Max ident
P43494.2	Rhodocoxin reductase	89%	4.00E-43	48%
P37337.2	Biphenyl dioxygenase system ferredoxin reductase	87%	2.00E-37	53%
Q07946.1	Benzene 1,2-dioxygenase system ferredoxin reductase	79%	3.00E-37	71%
P16640.1	Putidaredoxin reductase	89%	7.00E-35	46%
A5W4E9.1	Toluene 1,2-dioxygenase system ferredoxin reductase component	88%	2.00E-34	64%
B7N6C9.1	3-phenylpropionate dioxygenase ferredoxin reductase component	81%	2.00E-33	47%
P08087.3	Benzene 1,2-dioxygenase system ferredoxin reductase component	88%	2.00E-29	64%
Q9L4M8.1	Rubredoxin-NAD(+) reductase	75%	9.00E-28	43%
P33009.1	Terpredoxin reductase	72%	2.00E-24	55%

ipfI

Blast-X analysis against the Swissprot database revealed significant homology to a single characterized plant-like 2Fe-2S ferredoxin protein (P23263.1), part of a salicylate hydrogenase complex and a member of the plant-like ferredoxins, so called because such proteins play a role in chloroplast electron transport chains and are also commonly parts of aromatic dioxygenase complexes.

Ipf pathway overview

While complementation of *ipfF* was accomplished by cloning in the gene with the native ribosomal binding site (Table 3.2), attempts to complement the other transposon mutants were unsuccessful. In order to minimize the possibility of polar effects, markerless gene deletions were introduced into pFOS3G7 using a modified lambda red protocol. Each of the deletions failed to produce catechols (Figures 3.7 and 3.8). Because an initial attempt to complement pFOS3G7Δ*ipfD* using the native ribosomal binding site failed, complements were constructed using a forward primer that introduced a three frame stop codon followed by a strong conserved ribosomal binding site (Figure 3.2) in a high copy number vector under the *lacZ* promoter of the high copy number

vector pGEMt-easy. Complementation of pFOS3G7Δ*ipfD* with pGEM:*ipfD*rbs restored activity.

The cooperation of an SCPx thiolase (IpfD) and a DUF35 protein (IpfE) is not without precedent. In the benzosuccinyl-CoA pathway of *Thauera aromatica*, an SCPx and a DUF35 coordinate together to perform a beta-ketothiolytic decondsation (Figure 3.17d). Two similar genes are also involved in a polyketide aromatic acetylation in *P. putida*. While these two situations represent the only well-characterized reactions catalyzed by either type of protein in bacteria, examination of the NCBI database clearly shows that such genes are present in many bacteria; over 2000 putative SCPx thiolases and over 1500 DUF35 encoding genes have been identified by sequence analyses. There are also at least 43 cases in which these two gene types are located directly adjacent to one another in bacteria. In animals, SCPx proteins have been shown to catalyze the removal of propionyl-CoA from alpha-methyl-beta-keto coenzyme A fatty acids via a classical thiolytic reaction mechanism (Figure 3.15)(Wanders, Denis et al. 1997; Verhoeven and Jakobs 2001; Takeuchi, Chen et al. 2004; Westin, Hunt et al. 2007). The metabolite that IpfDE is predicted to act upon is dearomatized 1,2-diol-ibuprofen-CoA, an alpha-methyl-beta-hydroxyl CoA fatty acid. Similarly, IpfDE is predicted to remove a propionyl-CoA group (Figure 3.20).

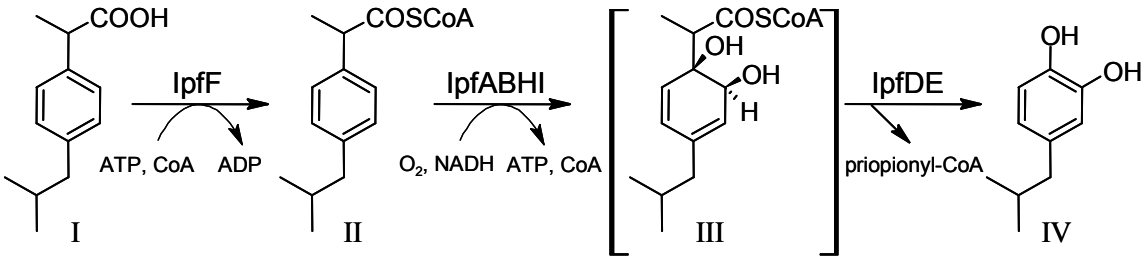

Figure 3.20. Proposed pathway by which Sphingomonas Ibu-2 metabolizes ibuprofen to isobutylcatechol. I: Ibuprofen, II: Ibuprofen-CoA, III: 1,2-*cis*-diol-2-hydroibuprofen-CoA, IV: 4-isobutylcatechol. The creation of II is suggested by the fact that IpfF was shown to CoA-ligate ibuprofen in an enzyme assay and by the analagous CoA ligation of phenylacetic acid as confirmed by HPLC analysis (discussed in Chapter 4). Additionally, the creation of phenylacetyl-coenzyme A, catalyzed by the same enzyme, was confirmed via HPLC. III has not been detected and is hypothesized based upon the putative function of IpfAB. The identity of IV was confirmed via GC/MS.

However, the predicted reaction mechanism does not allow for a thiolytic decondensation due to the mechanistic constraint caused by bond limitations of the ring beta carbon. Consistent with the requirement for a unique mechanism, IpfD does not have the amino acid residues associated with thiolase activity (Figure 3.18). The DUF35 proteins are predicted to have an affinity for acyl-CoA groups based on crystal structure analysis (Krishna, Aravind et al. 2010) and may serve a function similar to that played by sterol carrier protein 2 (SCP-2) in eukaryotes. SCP2 is an acyl carrier closely associated in function with SCPx proteins (Gallegos, Atshaves et al. 2001; Stolowich, Petrescu et al. 2002). SCP2 domains are not found in bacteria while DUF35 domains are not found in eukaryotes, a fact which suggests the possbility of convergent evolution.

Expressing *ipfDE* in *E. coli* K12 Δ*paaG* caused an increase in the amount of the dead-end metabolite 2-hydroxyphenylacetate that was produced (Figure 3.14). *E. coli* K12 Δ*paaG*, which lacks an isomerase capable of dearomatizing the phenylacetyl ring, accumulates 1,2-epoxyphenylacetyl-CoA which is rapidly abiotically degraded primarily

to 2-hydroxyphenylacetate (Figure 1.8)(Teufel, Mascaraque et al. 2010). Since expression of *ipfDE* did not increase 2-hydroxyphenlacetate levels in the wildtype or in the knockouts of the two downstream steps *paaZ* and *paaJ,* it is likely that the epoxy metabolite which is the substrate of PaaG is interacting with IpfDE in some manner. For many years, it was suspected that the bacterial phenylacetyl coenzyme A pathway involved a 1,2-dihydroxy intermediate which IpfDE would be hypothesized to convert to catechol by analogy to the *ipf* pathway (Ismail, Mohamed et al. 2003). The fact that we could not detect catechol via ferric chloride assay or by HPLC in the *paaG* mutant expressing *ipfDE* is consistent with recent evidence of the involvement of an epoxide intermediate in the *paa* pathway. The increased product of 2-hydroxyphenylacetate, however, suggests that there may be some interaction between IpfDE and the putative epoxyphenylacetyl-CoA; perhaps the epoxide is similar enough to the predicted 1,2-dihydroxyl intermediate that IpfDE is hypothesized to act upon that an interaction occurs in such a way as to either speed the hydrolysis of the epoxide or alter reaction kinetics in such a way that further production of the labile epoxide is favored. While inconclusive, this data is consistent with the involvement of IpfDE in the *ipf* and PAA degradation pathways in Ibu-2.

As discussed in more detail in Chapter 4, the predicted amino acid sequence of *ipfF* has limited similarity to a wide variety of coenzyme A ligases, including that from the Fad operon of *E. coli,* and to several ligases used in the anaerobic metabolism of benzoic acid (Table 4.2). IpfF showed no similarity to human xenobiotic/medium-chain fatty acid:CoA ligase which has been shown to be capable of performing ibuprofen CoA ligation (Vessey, Hu et al. 1996). That *ipfF* encodes for ligase activity of ibuprofen and

phenylacetate but not an oxidized intermediate is strongly supported by the complete and complementable lack of ibuprofen disappearance in the HPLC assay of *E. coli* epi300 pFOS3G7Tn:*ipfF* while all other loss-of-function clones retained some initial metabolic activity (Figure 3.6). Indeed, an initial coenzyme A ligation is the case in characterized fatty acid metabolic systems and in the phenylacetic acid catabolic pathway of *P. putida* U (Garcia, Olivera Elias et al. 2000; Teufel, Mascaraque et al. 2010).

Ibuprofen degradation in *E. coli* harboring pJ25 was very poor when compared with *E. coli* epi300 pFOS3G7, even though *ipfABDEF* expression was under the control of the *lac* promoter. Only trace amounts of catecholic metabolites were detected. One explanation for the reduced activity encoded by pJ25 is the need for other gene products encoded by pFOS3G7 that facilitate metabolism. Indeed, preliminary sequence analysis of non-loss-of-function pFOS3G7 transposon mutants revealed a putative ferredoxin (*ipfI*) *and* a ferredoxin reductase (*ipfH*). HPLC analysis from a knockout of the putative ferredoxin reductase (*ipfH*) in *E. coli* epi300 pFOS3G7 showed decreased ibuprofen disappearance though catechols are still generated (Appendix 2, Figure A2.2). Cloning and expressing these genes along with the *ipfABDEF* cassette found on pJ25 resulted in successful reconstruction of the *ipf* upper pathway, with 0.37 mM phenylacetate being converted to catechol within 18 hours (Figure 3.12) and approximately 0.25 mM ibuprofen being converted to isobutylcatechol (Figure 3.13). The dependency of the *ipfABDEF* pJ25 cassette upon the co-expression of *ipfHI* is consistent with the fact that many ring-hydroxylating aromatic dioxygenases require a reductase component for activity (Mason and Cammack 1992; Butler and Mason 1997) and offers circumstantial

support for the involvement of *ipfAB,* a putative aromatic ring dioxygenase, in the pathway.

While the initial data suggest that *ipfAB* are required for metabolism, we were unable to elucidate the mechanisms by which they function or any further metabolic intermediates. When *ipfAB* was cloned and expressed in *E. coli* and concentrated cell-free extract was exposed to phenylacetyl-CoA, the disappearance of phenylacetyl-CoA was significantly higher than in the control extract (161 vs 123 nM phenylacetyl-CoA / mg protein * hr, Figure 3.10, $p<0.05$). The backround disappearance of the phenylacetyl-CoA in the control, possibly due to abiotic hydrolysis or non-specific enzymatic activityr, makes it difficult to draw any firm conclusions about the role of IpfAB, especially given that GC/MS analysis failed to identify any reaction products in crude-extracts incubated with phenylacetyl-CoA.

It is not clear what roles IpfD and IpfE are playing. pFOS3G7-harboring clones with deletions of either of these genes were clearly unable to generate isobutylcatechol (Figure 3.12). However, attempts to identify accumulated intermediates via HPLC or GC/MS proved fruitless. Chromatographic analysis of these mutants with the addition of ^{14}C-ring-labelled ibuprofen or phenylacetic acid might allow for identification of any metabolite or byproduct that may be accumulating. While preliminary biochemical evidence and putative function implied from sequence similarity suggest that IpfAB dioxygenates ipfCoA and that IpfDE catalyze removal of the acyl-CoA group (Figure 3.20), these reactions warrant more investigation.

The removal of the acidic side-chain and associated oxidation of the 1-position of the aromatic rings of ibuprofen and phenylacetic acid are reminiscent of the removal of

an acyl group during *beta*-oxidation of fatty acids (Eaton 1996; Trotter 2001) such as is performed by the well-characterized Fad operon of *E. coli* (Black, DiRusso et al. 1992; Kunau, Dommes et al. 1995; Campbell and Cronan Jr. 2002). This work reveals that ibuprofen and other phenylacetic acids, a class of chemical that includes many of the non-steroidal anti-inflammatory pharmaceuticals, may be metabolized by a novel type of pathway involving coenzyme A ligation, removal of the acidic side-chain, and generation of a catechol through the acitivty of a thiolase/acyl-transferase and an aromatic dioxygenase.

All sequences can be found in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) nucleotide sequence database under accession number EF090268.

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CHAPTER 4

UPTAKE OF PHENYLACETIC ACID BY *SPHINGOMONAS IBU-2* FOSMID LIBRARY CLONE 3G7

Abstract

Sphingomonas Ibu-2 chromosomal DNA fosmid clone 3G7 (*E. coli* epi300 pFOS3G7) exhibits the novel ability to metabolize ibuprofen to isobutylcatechol and phenylacetate to catechol. The mechanism by which phenylacetate enters *E. coli* epi300 pFOS3G7 was investigated by radiolabelled uptake assays with special attention paid to the putative ibuprofen and phenylacetate coenzyme A ligase *ipfF*. *Sphingomonas* Ibu-2 exhibited constituitive phenylacetyl coenzyme A ligation activity. pFOS3G7 was also shown to confer phenylacetate coenzyme A ligation. When cloned into *E. coli* a high-copy number *ipfF* expression construct conferred a 68-fold increase in the rate of phenylacetate coenzyme A ligation and also catalyzed the coenzyme A ligation of ibuprofen. Uptake of phenylacetic acid by *E. coli* harboring pFOS3G7 was found to be enhanced by *ipfF*; when *ipfF* was knocked out, uptake was decreased two- to eight-foldUptake by *E. coli* epi300 pFOS3G7 was reduced to *ipfF* knockout levels following incubation with the metabolic poisons potassium cyanide or 2,4-dinitrophenol, although residual uptake by the *ipfF* knockout was unaffected by metabolic poisons, indicating that no other energy-dependent process was involved in

phenylacetic acid uptake in this fosmid clone Uptake of phenylacetic acid was greater at pH 5.5 than at pH 7. *In silico* analyses of the putative product of *ipfF* predicted the presence of a small membrane-associating helix, suggesting that the coenzyme A ligase might transiently bind to the cell membrane in a manner similar to that shown by the fatty acid coenzyme A ligase FadD which drives the uptake of fatty acids via vectorial acylation. These results are consistent with a coenzyme A ligation driven vectorial acylation mechanism for uptake of phenylacetic acid and ibuprofen which is more typical of fatty acid uptake and differs markedly from other recently described mechanisms for phenylacetic acid uptake which rely on symporters.

Introduction

Ibuprofen (2-(4-isobutylphenyl)-propionic acid) is a very widely used over the counter pharmaceutical compound which is self-administered primarily as an anti-inflammatory and pain reliever. It is consumed and excreted in sufficiently large quantities to be routinely detected in sewage treatment effluents and surface waters world-wide (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002). Compounds like ibuprofen that are not technically recalcitrant but which are continuously released into the environment are considered to be “pseudopersistent (Daughton 2002)”, like many other pharmaceuticals that enter the environment regularly from municipal sewage treatment plants. The environmental implications of this extremely diverse soup of biologically active compounds flowing into surface waters are not completely understood (Stuer-

Lauridsen, Birkved et al. 2000; Heberer 2002; Jones, Voulvoulis et al. 2004; Cunningham, Buzby et al. 2006; DeLange, Noordoven et al. 2006; Fent, Weston et al. 2006; Hernando, Mezcua et al. 2006; Schwarzenbach, Escher et al. 2006; Dorne, Skinner et al. 2007; Kummerer 2008; Kummerer 2010; Santos, Araújo et al. 2010). In order to begin to characterize the hazards associated with these pseudopersistent compounds more data is needed regarding their possible biological transformations and other aspects of their environmental fate.

This chapter describes attempts to elucidate the mechanism by which Ibu-2 transports ibuprofen and phenylacetic acid into the cell. Uptake is an essential aspect of any catabolic system as it is an obvious prerequisite for induction and further metabolism of the chemical in question (constitutive expression is not typically used in the case of most catabolic systems (Madigan and Martinko 2006)). Below a certain threshold, a chemical will tend to persist due to a combination of physical mass transfer (Bosma, Middledorp et al. 1997) and biological factors (Schmidt, Alexander et al. 1985). As concentration increases, the threshold is eventually reached where the chemical is transported into the cell where it will accumulate until the concentration is sufficient to trigger induction (Louis and Becskei 2002). Induction, somewhat non-intuitively, is seldom an analog process; it has been demonstrated that induction systems tend to be functionally binary in that below a particular threshold, no transcription takes place and when the threshold is crossed, transcription springs into action. It is partially due to the binary nature of the induction process that chemicals can persist to a surprising degree at low concentrations. For example, it has been demonstrated that metabolism of 3-phenylpropionic acid, an aromatic acid somewhat similar to ibuprofen, by *E. coli* is not

induced at concentrations below 3 mg/L (Kovar, Chaloupka et al. 2002). On the other hand, it has been demonstrated that phylogenetically undefined sewage and oligotrophic lake samples can mineralize aromatic acids at concentrations as low as a few ng/L (Rubin, Subba-Rao et al. 1982). In part because transport is an integral part of the induction process, understanding transport may be of assistance in predicting the environmental fate characteristics of a particular chemical.

Aromatic acids, including phenylacetic acid (Olivera, Minambres et al. 1998), phthalate (Chang, Dennis et al. 2009), benzoate (Clark, Momany et al. 2002; Ledger, Flores-Aceituno et al. 2009; Wang, Xu et al. 2011), chlorobenzoate (Ledger, Flores-Aceituno et al. 2009), and 4-hydroxybenzoate (Ditty and Harwood 2002), tend to be taken up by major facilitator systems that couple uptake to chemiosmotic gradients (Marger and Saier 1993; Pao, Paulsen et al. 1998; Kahng, Byrne et al. 2000; Kasai, Inoue et al. 2001). There is also evidence for the involvement of ABC type transporter systems required for the uptake of phthalate (Hara, Stewart et al. 2009) and 3-hydroxyphenylacetate (Arias-Barrau, Sandoval et al. 2005). On the other hand, fatty acid uptake is at least partially driven by vectorial acylation processes that require no specific membrane channels but instead relies on diffusion of the substrate into the cytoplasm where it is then ligated to coenzyme A and thereby trapped inside the cell (Weimar, DiRusso et al. 2002; Black and DiRusso 2003; Zou, F. et al. 2003). Additionally, there is mounting evidence that the vectorial acylation process works in concert with the action of a TonB-dependent outer membrane transporter FadL in the case of fatty acid transport (Ferguson and Deisenhofer 2002; van den Berg, Black et al. 2004) and similar proteins

TodX and TbuX in the case of toluene (Wang, Rawlings et al. 1995; Kahng, Byrne et al. 2000).

Although an aromatic acid, ibuprofen's four-carbon aliphatic side-chain and *alpha* methyl group also make it similar to fatty acids. In fact, analysis of the octanol water portioning coefficient of ibuprofen using the structure activity software KowWin in the program Episuite (Agency 2000) suggests that the majority of the hydrophobicity of ibuprofen comes from the aliphatic carbons. That Ibu-2 treats ibuprofen as a fatty acid is suggested by the catabolic processes that have been demonstrated to be involved or are predicted based on the gene sequences of the *ipf* operon; coenzyme A ligation and oxidation of the beta carbon followed by the action of a thiolase-type protein. Given that ibuprofen is metabolized in a manner similar to that by which fatty acids are metabolized and given the hydrophobicity of the ibuprofen molecule, I hypothesized that it is transported into the cell using a fatty acid-like mechanism driven by vectorial acylation. In this study, transport assays using *E.coli* heterologously expressing *ipfF* was employed to examine the roles played by these different genes in the uptake of ibuprofen and phenylacetic acid by Ibu-2.

Methods

Unless otherwise noted, chemicals were purchased from Acros (Morris Plains, NJ). Luria-Bertani broth (LB) was prepared as previously described (Sambrook, Fritsch et al. 1989). Phenylacetyl CoA was purchased from Sigma (St. Louis, MO) and coenzyme A was purchased from Roche Diagnostics (Indianapolis, IN).

Strains and Clones: Construction and Identity

ipfF was PCR amplified using the primers ipfFF and ipfFR (Table 3.2). The resulting PCR product was cloned into pGEMt-easy (Promega Corp., Madison, WI) using the manufacturers instructions, creating pGEM:*ipfF*. pGEM:*ipfF* was transformed into E. coli JM109 via electroporation. Successful clones were selected on LB ampicillin with blue/white screening via X-gal and 1mM IPTG then sequenced.

Table 4.1. Strains and plasmids used in this study.

Strains		
Sphingomonas Ibu-2	isolated from Ithaca, NY sewage treatment plant via enrichment for growth on ibuprofen	this study
E. coli JM109	recA1 subE44 endA1 hsdR17 gyrA96 relA1 thi Δ*(lac-proAB*) F' (*traD36 proAB+ lacIq lacZ ΔM15*)	(Sambrook, Fritsch et al. 1989)
E. coli epi300	F$^-$ *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ$^-$ rpsL (Str^R) nupG trfA tonA*	Epicentre, Madison, WI

Plasmids		
pCC1FOS	copy control fosmid vector, inducible to high copy number with arabinose when hosted in *E. coli* epi300, chloramphenicol resistance	Epicentre, Madison, WI
pFOS3G7	pCC1FOS *Sphingomonas* Ibu-2 chromosomal library clone 3G7	this study
pFOS3G7Tn:*ipfA*	pFOS3G7 with Tn5 insertion in *ipfA*, transposon library clone F1, chloramphenicol and tetracycline resistant	this study
pFOS3G7Tn:*ipfF*	pFOS3G7 with Tn5 insertion in *ipfF*, transposon library clone F10, chloramphenicol and tetracycline resistant	this study
pGEMt-easy	ampicillin resistance	Promega, Madison, WI
pGEM:*ipfF*	pGEMt-easy with ipfFF/ipfFR PCR amplicon ipfF	this study

Coenzyme A ligase activity

Wild type Ibu-2 and *E. coli* harboring either pFOS3G7 or various subclones were assayed for phenylacetyl coenzyme A ligase and ibuprofen coenzyme A ligase activity. *P. putida* U was also used as a positive control because it is known to perform phenylacetyl coenzyme A ligation (Martinez-Blanco, Reglero et al. 1990). The inducibility of phenylacetyl coenzyme A ligase activity in Ibu-2 was tested by growing two batches of Ibu-2 in LB and adding 50ppm ibuprofen to one for one hour prior to preparation of cell-free extract.

The method described by Martinez-Blanco *et al.* (Martinez-Blanco, Reglero et al. 1990) was used with minor modifications for measuring phenylacetyl coenzyme A ligase activity and ibuprofen coenzyme A ligase activity. In the case of Ibu-2 and *P.putida* U, crude extract was prepared by first concentrating 100mL of *E. coli* or a 1000mL of *P .putida* or Ibu-2 culture via centrifugation, followed by two washes with 10 mM phosphate buffer (pH 7.4) and resuspension in 1-2 ml of sonication buffer (40 mM potassium phosphate, 20% glycerol, 1mM PMSF, 1mM DTT, pH 7.4). Larger volumes of *P. putida* and Ibu-2 cultures were used due to the lower overall culture density yielded by growth on minimal media. *E. coli* cultures were grown in LB media with IPTG or arabinose for induction, *P. putida* was grown in MSM with 5mM PAA, and Ibu-2 was grown in MSM with 500 mg/L ibuprofen. The cell pellet was then sonicated (Branson Sonifier 450, Branson Ultrasonics, Danbury, CT) using three one minute cycles at maximum output with one minute rest time on ice in between each cycle. The cell lysate was then spun for twenty minutes at 15,000 x g at 4° C. In the case of *E. coli* clones, crude extracts were prepared by centrifuging 100ml to 200ml of mid-exponential growth

phase of either IPTG or 10mM arabinose induced culture, washing, and resuspending in 1-2 ml of sonication buffer. Approximately 0.1g of 0.1 mm glass beads was then added and the pellets were bead-beaten for three minutes (MiniBeadbeater-8, Biospec Products, Bartlesville, OK). The pellets were then spun down as described above. In both cases, the protein content of the supernatant containing the crude extract was quantified using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA).

The reaction mixture for Co-A ligase assays included 4.2 µl 0.2 M $MgCl_2$, 17 µl 0.1 M ATP, 10 µl 20 mM coenzyme A, 10 µl 0.1 M phenylacetic acid or ibuprofen, 20 µl cell-free extract (~0.1 mg of crude protein), and 17 µl neutral hydroxylamine solution. Neutral hydroxylamine solution was prepared freshly by mixing 1 ml of 5 M hydroxylamine hydrochloride, 1.25 ml 4 M KOH, and 250 µl of water. 150 µl ferric chloride reagent (0.37 M ferric chloride, 20 mM trichloroacetic acid, and 0.66 M hydrochloric acid) was added to terminate the assay at the prescribed time. Samples were spun down to remove precipitate, and the OD540nm was measured using a µ-Quant spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Concentrations of the phenylacetate ferric chloride complex were calculated using the known extinction coefficient of 0.9 /(mM)(cm) (Martinez-Blanco, Reglero et al. 1990). Because the extinction coefficient of the ibuprofen ferric chloride complex (putatively derived from ibuprofen-CoA) is unknown, the phenylacetate ferric chloride value was used.

The disappearance of phenylacetic acid and concomitant appearance of phenylacetyl-CoA was also measured via HPLC using the methods described by Mohamed *et al.* (Mohamed and Fuchs 1993). The reactions were performed as listed

above except for the exclusion of neutral hydroxylamine and the addition of an equimolar amount of coenzyme A. For the detection of phenylacetyl-CoA (retention time 45 minutes), the running solvent was 30mM potassium phosphate buffer pH7 with 9% methanol and 7% acetonitrile. Phenylacetic acid was analyzed using 50% 40mM acetic acid and 50% methanol and eluted from the column at 10 minutes. Phenylacetic acid was monitored at 210 nm and the appearance of phenylacetyl CoA was monitored at 254 nm.

Uptake in Ibu-2 and E. coli constructs

To examine the contribution of certain genes to uptake rates of Ibu-2, *E. coli* epi300 pFOS3G7, and *E. coli* epi300 pFOS3G7 with a transposon insertion in *ipfF* were selected for uptake assays. To determine if the impact of this gene product was concentration dependent, a range of phenylacetic acid concentrations was used; 0.5μM, 2.5μM, 25μM, 100μM, 500μM, and 1mM.

Effect of metabolic poisons on phenylacetic acid uptake by E. coli epi300 pFOS3G7*, and E. coli epi300 pFOS3G7Tn:ipfF*

Metabolic poisons were utilized to examine the energy-requirement of the uptake system. Two poisons with different modes of action were used; potassium cyanide and 2,4-dinitrophenol (2,4-DNP). Poisoning assays were performed on *E. coli* epi300 pFOS3G7 and *E. coli* epi300 pFOS3G7Tn:*ipfF* using a phenylacetate concentration of 25μM. The assays were performed as described above, except that after the initial wash step, the cells were suspended in 0.5 mM KCN, 0.5 mM DNP, or no poison, all in MSM

for 10 minutes. The cells were then centrifuged again, resuspended with the radiolabeled substrate, and the assay proceeded as described above.

Effect of pH on uptake by E. coli epi300 pFOS3G7

The impact of pH on phenylacetic acid uptake was examined in *E. coli* epi300 pFOS3G7. If the solute was merely diffusing across the membrane, then lowering pH should increase the degree of uptake due to the protonation of the acid group, thereby eliminating its negative charge and facilitating passage of the neutral molecule into the biological membrane. A wide range of phenylacetic acid concentrations was examined, from 0.5 uM to 1 mM. A low pH (5.5) and neutral pH (7) were used. The assay was performed as described above except that the solution of radiolabelled phenylacetic acid MSM was adjusted to the desired pH with 1M HCl.

Uptake Assays

Techniques for uptake assays were adapted from those described by Leveau *et al.* (Leveau, Zehnder et al. 1998). Ibu-2 was inoculated (5% V/V) in MSM with 500 ppm ibuprofen using a late exponential growth phase culture and grown for approximately 24 hours, which allowed for harvest of cells in mid-exponential growth phase. All the *E. coli* strains were similarly inoculated (5% V/V) into LB with appropriate antibiotics using overnight cultures, then induced to high-copy number using 10mM arabinose and grown for 5 hours. Cultures were centrifuged and washed with MSM pH 7.4 once before being centrifuged again and then resuspended to an approximate optical density (600nm) of one in MSM.

For each data set, 50,000 dpm of ^{14}C phenylacetic acid was added to 3mL MSM containing twice the desired concentration of unlabeled phenylacetic acid at pH 7.4. To initiate the uptake assay, 3mL of washed cell suspension was added to the 3mL phenylacetic acid solution. The resulting 6mL solution was well-mixed and then filtered in 2mL aliquots at the appropriate time. The filter apparatus consisted of a 6mL plastic syringe attached to a reusable filter cartridge preloaded with a 0.45 micron filter membrane. At one minute, the cell suspensions were forced through the filter. Each membrane was then washed with 2mL of 50 uM phenylacetic acid MSM solution to displace any labeled surface-bound phenylacetic acid. The filters were then removed, placed in scintillation tubes containing 5mL scintillation cocktail, and measured in a scintillation counter. The fraction of ^{14}C phenylacetic acid retained on the membranes was assumed to equal the fraction of total phenylacetic acid taken up by the cells.

Phenylacetic acid uptake was standardized to protein content of the cell suspensions. Each sample was centrifuged and the protein quantified via the Bradford assay (Bio-Rad Laboratories, Hercules, CA) after they had been boiled in 0.1 M NaOH for ten minutes.

In silico analyses of ipfF

Phylogenetic analysis of the putative gene product of *ipfF* was performed with the COBALT multiple alignment tool (Papadopoulos and Agarwala 2007) using Fast Minimum Evolution tree building method with Grishin distance. The putative protein product of *ipfF* was aligned with paaK from *E. coli* (NCBI BAE76428.1), the recently crystallized paaK1 and paaK2 from *Burkholderia cenocepacia* (PDB 2Y27 and 2Y4O),

and FadD fatty acid coenzyme A ligase from *E. coli* (NCBI P69451.1) in the MegAlign program (DNAStar, Inc.) using clustalW distance measure. Initial screening of proteins for transmembrane helices was performed using the PHDhtm algorithm (Rost, Casiado et al. 1995) provided by the PSIPRED server for definitive prediction of treansmembrane helices at 95% confidence level (Jones 2007) and using the TMHMM algorithm to provide redundancy of transmembrane helix detection and also to provide visual output (Krogh, Larsson et al. 2001).

Results

Assaying coenzyme A ligase activity

CoA ligase activity in Ibu-2 cell free extracts was determined using both phenylacetic acid and ibuprofen as substrates. Ibu-2 crude extract catalyzed phenylacetyl CoA ligation (Figure 4.1) at a rate of approximately 10 nmol/mg/minute, which was similar to that of *P. putida* U (data not shown). Ibu-2 extracts also catalyzed the CoA ligation of ibuprofen when compared to *P. putida* U ($p=0.007$), which did not yield detectable CoA-ligation product. However, the rate of ibuprofen CoA ligation by Ibu-2 was much lower; while almost all phenylacetic acid was ligated within two hours, only 33% (+/- 4%) of the ibuprofen was ligated in 63 hours. Prior exposure of Ibu-2 to Ibuprofen had no affect on CoA ligase activity ($p=0.36$) (Figure 4.1).

Crude extract from *E. coli* epi300 pFOS3G7 or *E. coli* JM109 pGEM:*ipfF* both contained detectable phenylacetyl CoA activity, 34 and 19.6 nmoles / mg protein / minute compared to 0.5 in vectorless epi300 (Figure 4.1). HPLC analyses confirmed that the

accumulation of phenylacetyl CoA was concomitant with the disappearance of phenylacetic acid (data not shown). Both phenylacetic acid and ibuprofen CoA-ligase activities were completely dependent upon the presence of ATP and Mg^{++} (data not shown).

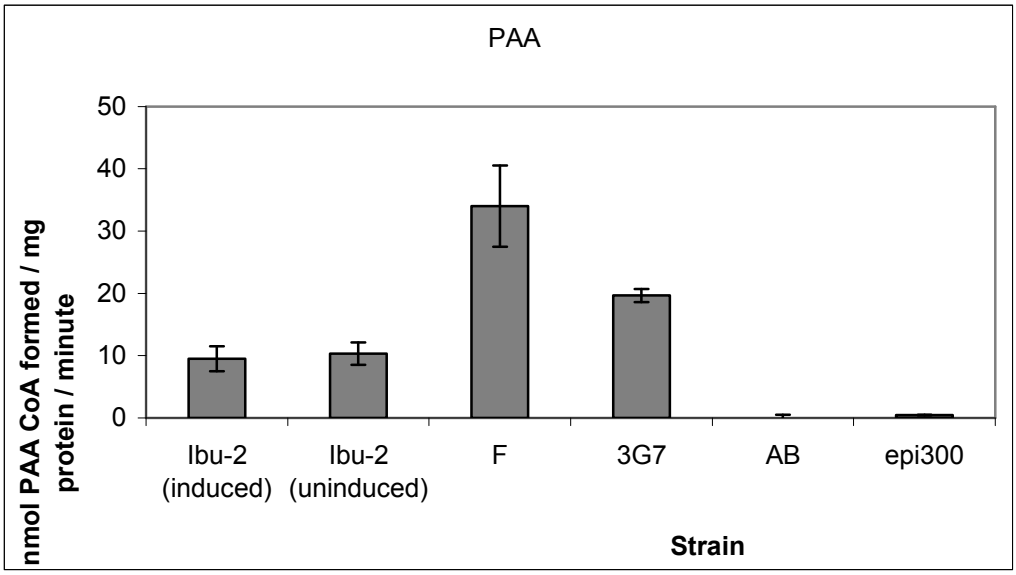

Figure 4.1. Phenylacetic acid coenzyme A ligase activity in the cell-free extracts of different strains. "F" represents the pGEMt-easy *ipfF* clone while "AB" is the respective *ipfAB* clone. (n=3).

Uptake of phenylacetic acid in all strains, clones, and knockouts

The results detailed in chapter 3 demonstrated that *E. coli* epi300 pFOS3G7Tn*:ipfF* was not able to produce isobutylcatechol from ibuprofen. At all concentrations studied, *E. coli* epi300 pFOS3G7Tn*:ipfF* displayed less phenylacetic acid uptake than *E. coli* epi300 pFOS3G7 (Figure 4.2).

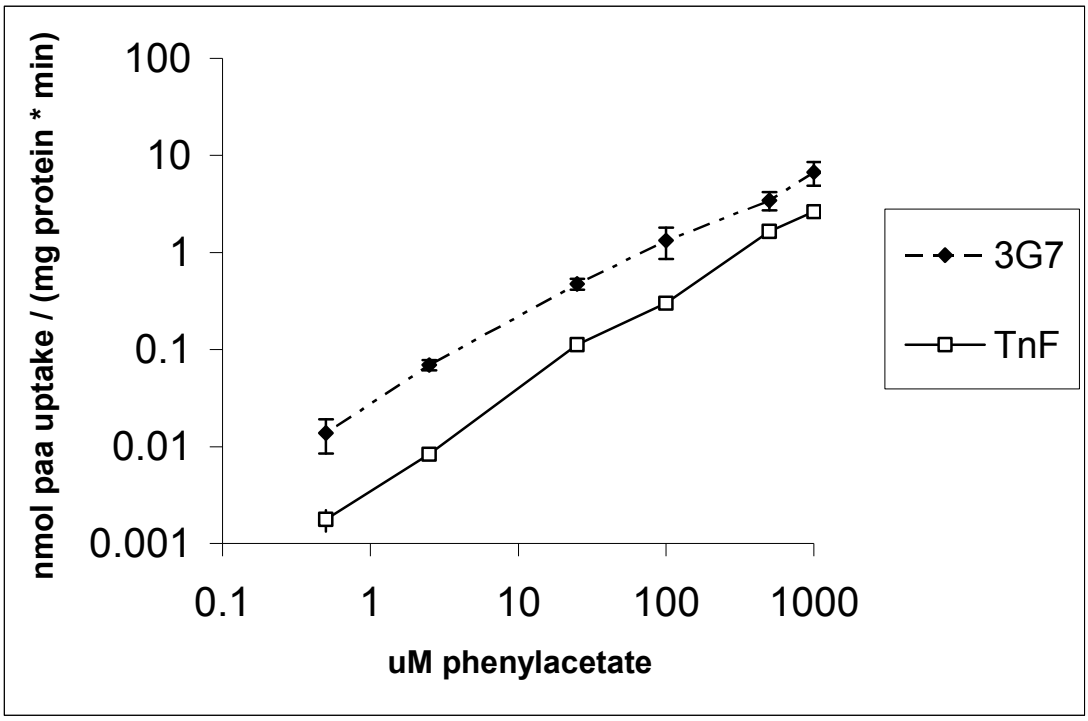

Figure 4.2. Uptake of phenylacetic acid by strains *E. coli* epi300 pFOS3G7, *E. coli* epi300 *E. coli* and epi300 pFOS3G7Tn*:ipfF* over a range of phenylacetic acid concentrations. Uptake was standardized to total cell protein. *E. coli* epi300 pFOS3G7Tn*:ipfF* was significantly less (n=3, p < 0.05) than *E. coli* epi300 pFOS3G7 at all concentrations tested.

Effect of metabolic poisons on E. coli epi300 pFOS3G7, and E. coli epi300 pFOS3G7Tn:ipfF

Uptake assays using Ibu-2 proved to be infeasible. Ibu-2 clogged up the filter membranes rapidly, prohibiting prompt filtration to any degree. This is likely due to its extensive exopolysaccharide layer which has been frequently observed in other Sphingomonads (Pollock 1993). The metabolic poisons significantly reduced phenylacetic acid uptake by *E. coli* epi300 pFOS3G7 (Figure 4.3). Uptake was reduced by approximately 23-32% by both poisons ($p<0.05$). Metabolic poisons did not affect uptake by *E. coli* epi300 pFOS3G7Tn*:ipfF* to any statistically detectable degree (Figure 4.3).

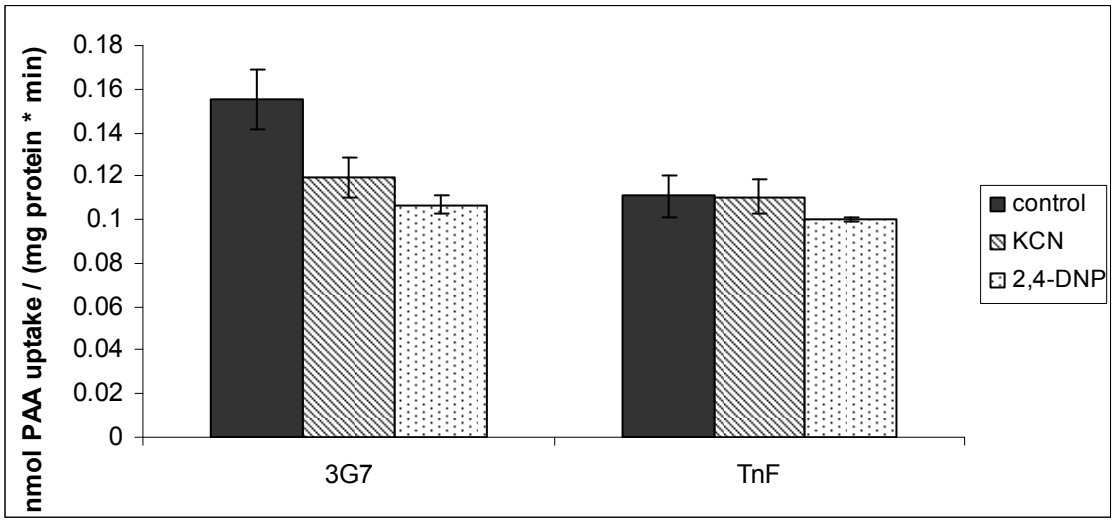

Figure 4.3. Uptake of 25 µM phenylacetic acid by *E. coli* epi300 pFOS3G7 and *E. coli* epi300 pFOS3G7Tn:*ipfF* exposed to 0.5mM of the metabolic poisons 2,4 dinitrophenol or potassium cyanide. Both pFOS3G7 poisoning treatments exhibited significantly less uptake than the non-poisoned control (n=3, $p < 0.05$) while no other differences were significant.

Effect of pH on uptake

The impact on uptake of phenylacetic acid by *E. coli* epi300 pFOS3G7 at a low vs. high pH was quantified by calculating the ratio of uptake at pH 5.5 vs. pH 7. At all concentrations other than the lowest, uptake was higher at low pH, indicating that diffusion across the membrane is a significant driving force in uptake.

The ratio of low/high pH uptake showing a trend of being proportional to the logarithm of phenylacetic acid concentration (best fit line R=0.8941, Figure 4.4). In other words, low pH had more of an impact on higher concentrations of phenylacetate, suggesting that pH drives transport to some degree but that there is also an underlying non-pH based transport mechanism at work. If no such mechanism existed, one would expect pH to alter uptake equivalently at all solute concentrations.

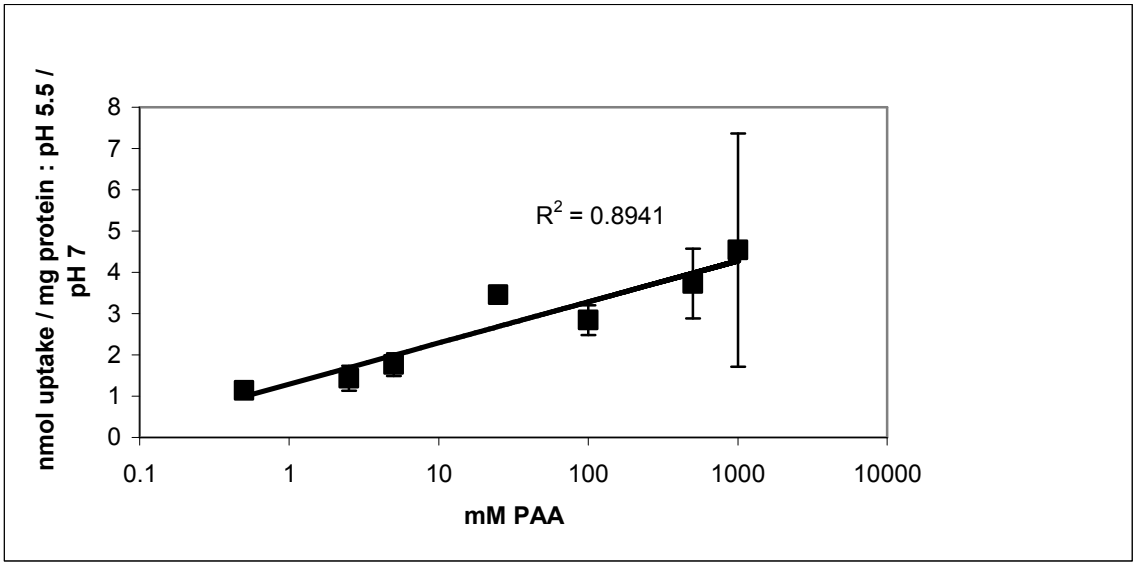

Figure 4.4. Phenylacetic acid uptake by *E. coli* epi300 pFOS3G7at pH 5.5 divided by uptake at pH 7. All data points other than for 0.5mM phenylacetate demonstrate that uptake was higher at pH 5.5 vs pH 7 as is consistent with the vectorial acylation mechanism (n=3, $p < 0.05$).

Discussion

ipfF was identified by transposon mutagenesis of pFOS3G7 and metabolic screening for loss of ability to convert ibuprofen to a catecholic metabolite as described in Chapter 3. *ipfF* had modest similarity to a number genes encoding coenzyme A ligases, particularly to those involved in degradation of aromatic acids with three (Achterholt, Priefert et al. 2000) and four (Veith, Herzberg et al. 2004) carbon acidic sidechains.

Conserved domain analysis of the predict amino acid sequence of *ipfF* revealed it to be a member of the LuxE superfamily (E-value = 1.64e-33), which harbor a domain associated with the formation of thioester bonds with representatives amongst all domains of life. The superfamily includes the coenzyme A ligases. A BlastP search of

IpfF against the Swissprot database reavealed similarities to a wide variety of coenzyme A ligases including those for aromatic acids and for fatty acids (Table 4.2). IpfF does not clearly group with any particular type of coenzyme A ligase; curiously, amongst the proteins with significant homology, it is most distantly related to the phenylacetyl CoA ligases (Figure 4.5) with which it shares only 40% similarity over 50-60% of the sequence, while sharing 45+% similar residues across 95+% of the sequence in the case of the long-chain fatty acid coenzyme A ligases from bacteria (Matsuoka, Hirooka et al. 2007) and even with 4-coumarate CoA ligase from the plant *Arabidopsis* (Ehlting, Battner et al. 1999) (Table 4.2).

Table 4.2. The substrates, similarity measures, and confidence levels between IpfF and characterized coenzyme-A ligases found in the Swiss-prot database.

Accession	Description (alpha subunit of) Organism	% identity	% positive	Query coverage	E value
O07610.2	long-chain fatty acid CoA ligase *Bacillus subtilis*	27	47	96%	2.00E-53
Q9S725.2	4-coumarate CoA *Arabidopsis thaliana*	29	47	98%	9.00E-51
P69451.1	long-chain fatty acid CoA ligase *E. coli* K12	26	45	96%	1.00E-47
AAC73148.2	crotonobetaine/carnitine-CoA ligase *E. coli* K12	28	46	94%	1.00E-43
Q00594.1	medium-chain fatty acid CoA ligase *Pseudomonas oleovorans*	25	47	96%	6.00E-39
YP_559588.1	benzoate CoA ligase *Burkholderia xenovorans*	27	45	93%	1.00E-35
AAN32623.1	benzoate CoA ligase *Thauera aromatica*	25	41	93%	5.00E-28
AAA92151.1	benzoate CoA ligase *Rhodopseudomonas palustris*	24	42	96%	5.00E-25
A5JTM6.1	4-chlorobenzoate CoA ligase *Pseudomonas* CBS-3	27	41	70%	2.00E-22
YP_931807.1	phenylacetyl CoA ligase *Azoarcus* sp.	24	41	60%	1.00E-12
CAA66100.1	phenylacetyl CoA ligase *E. coli* K12	25	40	57%	1.00E-11
YP_002768992.1	phenylacetyl CoA ligase *Rhodococcus erythropolis*	25	40	54%	2.00E-09
ZP_05785221.1	phenylacetyl CoA ligase *Silicibacter lacuscaerulensis*	23	40	55%	7.00E-08

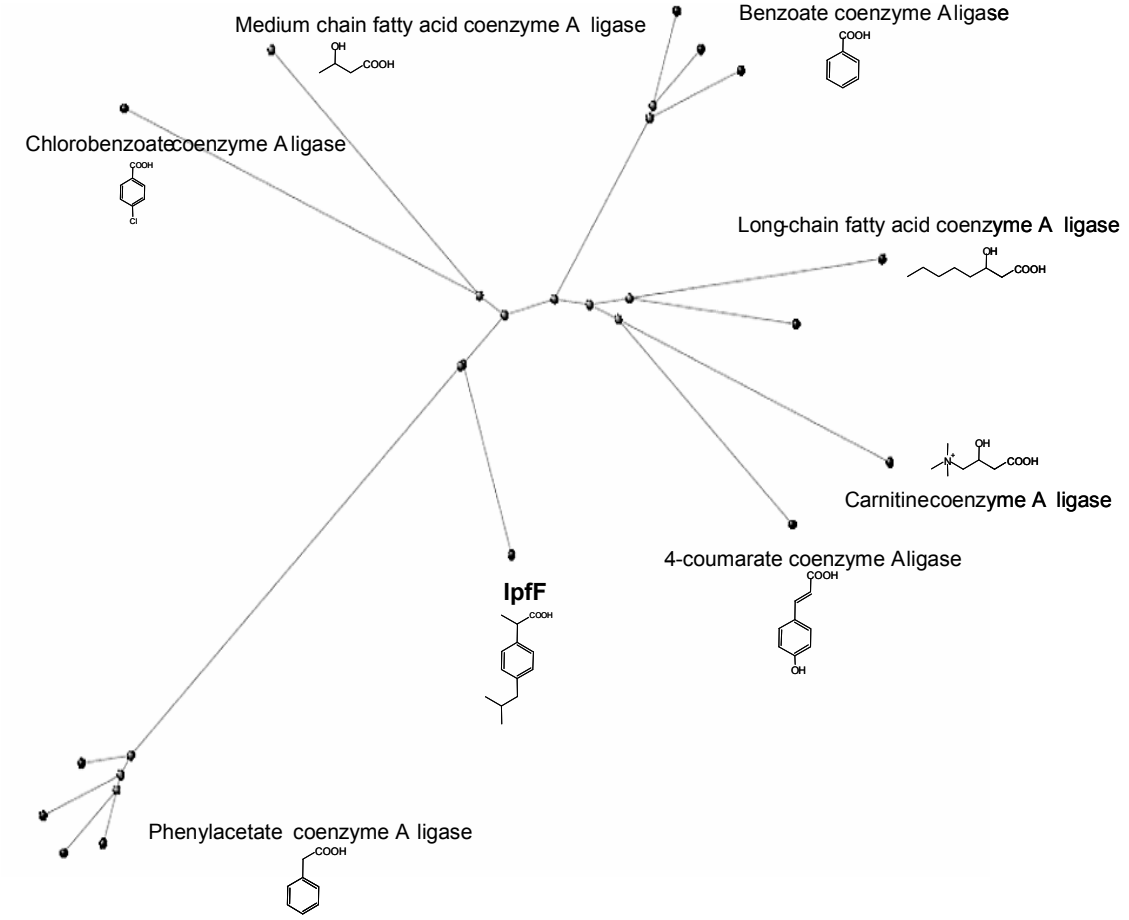

Figure 4.5. Phylogenetic tree of the predicted aminoacid sequence of *ipfF* and characterized coenzyme A ligases found in the Swiss-prot database and listed in Table 4.2.

Crude extracts from *Sphingomonas* Ibu-2 and *E. coli* epi300 pFOS3G7 were shown to possess phenylacetyl-CoA ligase activity (Figure 4.1). Activity was further shown to be completely dependent upon the presence of Mg^{2+} and ATP (data not shown). *ipfF*, which encodes the putative coenzyme A ligase, was further subcloned and expressed in *E. coli* where it was shown to confer both phenylacetyl- and ibuprofen-CoA ligase activity. This data establishes the role of IpfF in the first step of the *ipf* pathway as postulated in chapter 3 (Figure 3.17).

The mode of activity of *Sphingomonas* Ibu-2 ibuprofen transport gene products was studied via filter-based radio-labeled substrate uptake assays. Based on previous findings regarding the mechanism by which Ibu-2 catabolizes ibuprofen, an uptake system analogous to fatty acid uptake systems was hypothesized. It was previously demonstrated that the ibuprofen catabolic machinery (encoded by *ipfABDEF*) was capable of acting upon phenylacetic acid and similar compounds in the same way that ibuprofen was metabolized. Given its ready availability, ^{14}C phenylacetic acid was therefore used as a surrogate for ibuprofen.

The roles played by different *ipf* gene products on the uptake of PAA was studied with the aid of genetic constructs; *E. coli* epi300 pFOS3G7 is fully capable of metabolizing ibuprofen to the catechol stage, while transposon mutant of *ipfF* is fully defunct in this ability (Figure 3.6). The data presented in these figures is consistent with a model of ibuprofen uptake via fatty acid-like vectorial acylation. The core result supporting this model is the observation that *E. coli* epi300 pFOS3G7Tn*:ipfF* took up less phenyacetate than. *E. coli* epi300 pFOS3G7 at all substrate concentrations tested. This is consistent with previous findings that *E. coli* epi300 pFOS3G7Tn*:ipfF* was unable to cause the disappearance of ibuprofen. Heterologous expression of *ipfF* was shown to confer both ibuprofen and phenylacetate coenzyme A ligase activity on *E. coli*. Taken altogether, it is clear from these findings that *ipfF* encodes the first step in ibuprofen metabolism.

Phenylacetate uptake by *E. coli* epi300 pFOS3G7 was inhibited by the addition of either potassium cyanide or 2,4-dinitrophenol. Cyanide directly interferes with electron transport, thus slowing the pumping of protons outside of the inner bacterial membrane

(Gregus and Klaasen 2001). 2,4-DNP is a protonophore; it is believed to destroy the electrochemical proton gradient by serving as a functional proton conduit across the inner membrane. The impacts of both poisons relevant to this assay are the slowing of ATP production, which may be used in active transport, and the loss of the electrochemical proton gradient, which can be used to drive solute symport. This inhibition of uptake is fully consistent with the model of IpfF-catalyzed coenzyme A ligase activity driving uptake via vectorial acylation. Coenzyme A ligases are dependent upon energy provided by ATP (Kunau, Dommes et al. 1995; Vellemur 1995; Trotter 2001). If ATP production is inhibited by an uncoupler such as cyanide or a protonophore such as 2,4 dinitrophenol, ATP will be in short supply and the substrate that diffuses into the cell will not be trapped via acylation, rather, it will remain free to diffuse back out of the cell.

Alignment of the putative product of *ipfF* (IpfF) with three phenylacetyl coenzyme A ligases and FadD showed that IpfF has the conserved P-loop, an ATP-binding domain (Table 4.3). IpfF also has all three conserved residues associated with the first adenylation step catalyzed by coenzyme A ligases (Table 4.4). IpfF also contains five of the eleven residues that have been demonstrated to form the phenyacetate substrate binding pocket in PaaK1 (Law and Boulanger 2011) (Figure 4.5).

Table 4.3 Alignment, positions, and identities of ATP-binding P-loop residues

P-Loop Residues	
PaaK2 *B. cenocepacia*	^{100}SSGTTGKPT^{108}
PaaK1 *B. cenocepacia*	^{93}SSGTTGKPT^{101}
PaaK *E. coli*	^{98}SSGTTGKPT^{106}
FadD *E. coli*	^{214}YTGTTGVAK^{222}
IpfF *S.* Ibu-2	^{165}TSGTTGVPK^{173}

Table 4.4 Positions and identities of conserved adenylating catalytic residues

Catalytic Residues			
PaaK2 *B. cenocepacia*	Arg^{333}	Glu^{248}	Lys^{432}
PaaK1 *B. cenocepacia*	Arg^{327}	Glu^{242}	Lys^{424}
PaaK *E. coli*	Arg^{331}	Glu^{246}	Lys^{427}
FadD *E. coli*	Arg^{453}	Glu^{361}	Asp^{511}
IpfF *S.* Ibu-2	Arg^{410}	Glu^{309}	Lys^{510}

Table 4.5 Positions and identities of residues that align with the binding pocket residues of PaaK1 from *B. cenocepacia*. Residues that are identical to those found in PaaK are darkly-shaded while similar residues (as judged by charge) are lightly-shaded.

Binding Pocket Residues												
PaaK *E. coli*	Tyr^{141}	Phe^{146}	Gly^{148}	Ala^{152}	Gly^{218}	Ala^{219}	Ala^{241}	Tyr^{242}	Gly^{243}	Gly^{249}	Pro^{250}	Pro^{250}
PaaK2 *B. cenocepacia*	Phe^{143}	Phe^{148}	Gly^{150}	Ile^{154}	Gly^{220}	Ala^{221}	Ile^{243}	Tyr^{244}	Gly^{245}	Gly^{251}	Pro^{252}	Pro^{252}
PaaK1 *B. cenocepacia*	Tyr^{136}	Phe^{141}	Gly^{143}	Ala^{147}	Gly^{214}	Ala^{214}	Ile^{237}	Tyr^{238}	Gly^{239}	Gly^{245}	Pro^{246}	Pro^{246}
FadD *E. coli*	Pro^{158}	Phe^{264}	Leu^{265}	Cys^{269}	Gly^{333}	Ala^{334}	Gly^{256}	Tyr^{257}	Gly^{258}	Pro^{364}	Val^{365}	Val^{365}
IpfF *S.* Ibu-2	Pro^{204}	Trp^{209}	Val^{211}	Asn^{215}	Gly^{280}	Ala^{281}	Ala^{304}	Tyr^{305}	Ala^{306}	Gly^{312}	Ile^{315}	Ile^{315}

While PHDhtm predicted a single transmembrane motif for both FadD and the putative product of *ipfF*, the lengths of the putative membrane-spanning helices (8-14 residues) are likely too small to span a lipid bilayer (Cuthbertson, Doyle et al. 2005). True membrane-spanning helices tend to be 20-30 residues long (Cuthbertson, Doyle et al. 2005). TMHMM made similar predictions (Figure 4.6), with putative but low probability transmembrane-like motifs in the region of the amino acid chains where three conserved binding pocket motifs are located approximately 30-40 amino acids downstream of the ATP-binding P-loop domain. Neither program detected transmembrane motifs in PaaK1. These kinds of short transmembrane-like motifs have been termed “half-TMs” (Cuthbertson, Doyle et al. 2005).

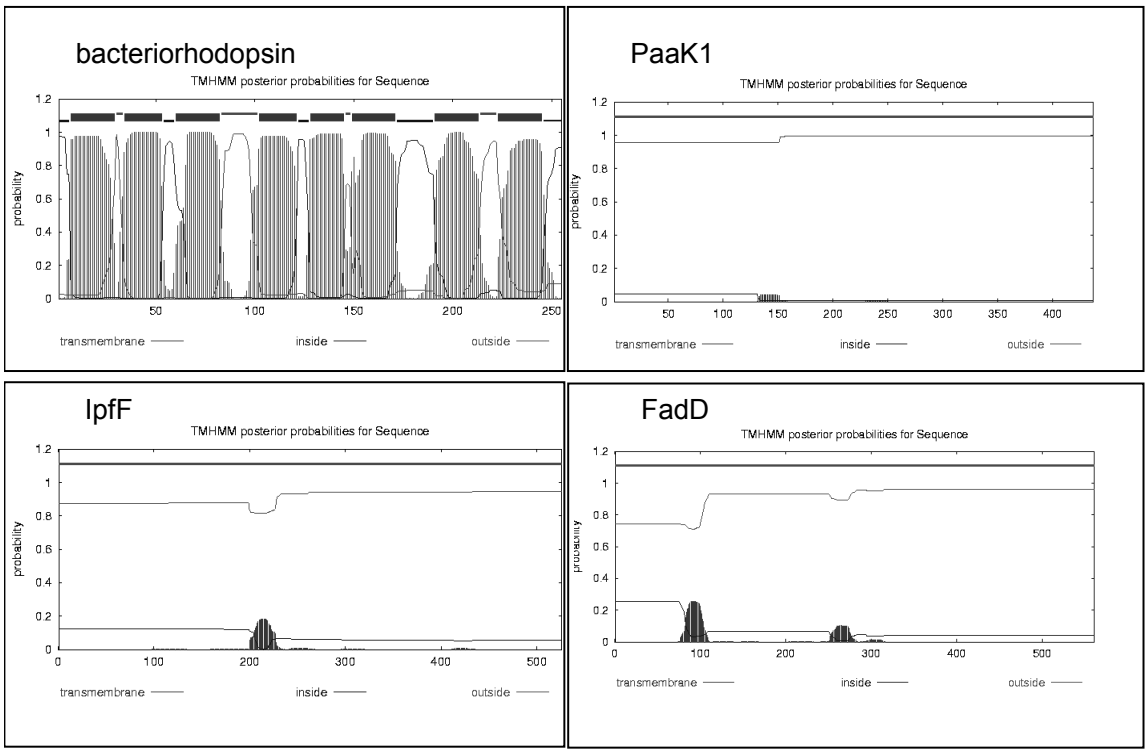

Figure 4.6 Location and probabilities of putative transmembrane helix motifs in PaaK1, IpfF, and FadD as predicted by the TMHMM algorithm (Krogh, Larsson et al. 2001). Bacteriorhodopsin, an extensively membrane associated protein (Haupts, Tittor et al. 1999), is presented for reference. Amino acid locations are shown on the x-axis while the probability of membrane association is represented on the y-axis.

It has been demonstrated that FadD associates with the membrane; both membrane and soluble enzyme fractions of induced *E. coli* K-12 contain FadD activity (Weimar, DiRusso et al. 2002). It has been hypothesized that free substrate dissolved in the membrane recruit FadD to become membrane-associated (Weimar, DiRusso et al. 2002), possibly involving a drastic conformational change common to this general class of proteins (Law and Boulanger 2011). This membrane association might be due to the short half-TMs present in each of these proteins, though there are other ways by which proteins can transiently associate with membranes, such as amphipathic helices and hydrophobic loops (Goni 2002). FadD has been shown to be sufficient to drive uptake of

fatty acids which diffuse into the inner bacterial membrane in *E. coli* and into the cytoplasm where they are trapped via coenzyme A ligation (Weimar, DiRusso et al. 2002) (Figure 4.7).

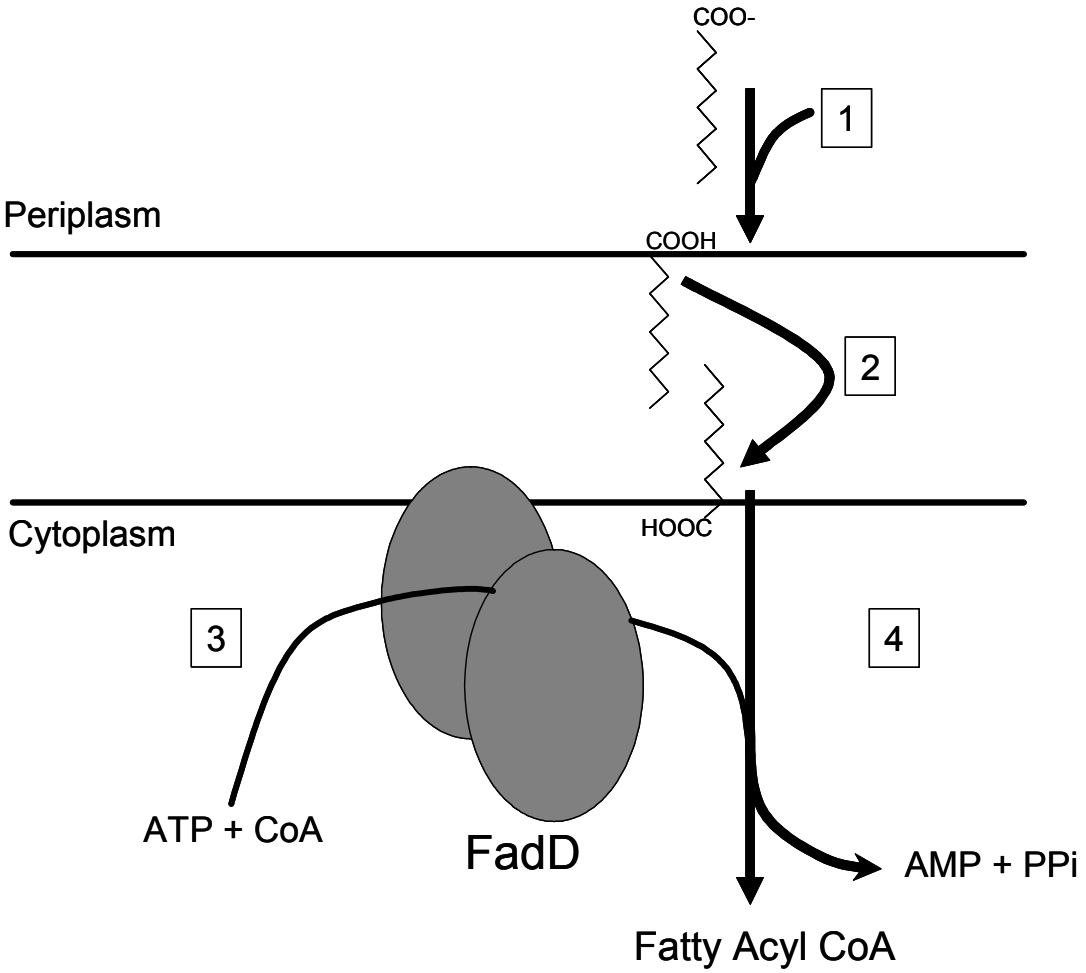

Figure 4.7 Model illustrating the role of FadD in the vectorial transport of exogenous fatty acids (Weimar, DiRusso et al. 2002). 1, free fatty acids become protonated in the periplasmic space following FadL-dependent transport across the outer membrane (not illustrated here) and then partition into the inner membrane. 2, the protonated fatty acid flips from the periplasmic face of the inner membrane to the cytoplasmic face. 3, free fatty acids within the membrane signal FadD to partition into the inner membrane, presumably in an ATP-bound form. 4, FadD functions to abstract fatty acids from the inner membrane concomitant with the formation of fatty acyl-CoA for further metabolism.

The similarity of membrane-associating regions present in both FadA and the putative product of *ipfF* are consistent with a similar vectorial acylation driven uptake system for ibuprofen and phenylacetic acid by the *ipf* pathway in *Sphingomonas* Ibu-2. The proximity of the putative half-TM (in the 200-220 residue range) to the ATP-binding P-loop (residues 165-173) and three of the residues that align with conserved binding pocket residues (Pro^{204}, Trp^{209}, Val^{211}, and Asn^{215}) is consistent with an IpfF model wherein a membrane-associating region binds with and adenylates membrane-dissolved substrate.

It would be possible to test this model by separating membrane-associated and cytoplasmic protein fractions and testing each for phenylacetate and ibuprofen coenzyme A ligase activity using the methods employed by Weimar *et al.* in their description of FadD membrane association (Weimar, DiRusso et al. 2002).

The vectorial acylation model is also consistent with the observation that the background uptake of phenylacetate by *E. coli* epi300 pFOS3G7Tn*:ipfF* was not affected by the addition of metabolic poisons.

It is important to keep in mind that this work was all performed using cloned genes in an *E. coli* host. Uptake assays could not be performed using *Sphingomonas* Ibu-2 because of the fact that the extensive exopolysaccharide clogged the filter membranes. While vectorial acylation may be the prime driving force in *E. coli* harboring *ipfF*, it may be the case that Ibu-2 requires more extensive machinery to take up ibuprofen due to membrane structure or exopolychaccharide layer differences. Cloning *ipfF* into a different Sphingomonad or another more closely related strain would begin to address the question of whether such species specific factors are important to the transport system.

While benzoate and phenylacetate coenzyme A ligases have been described, in each of these cases traditional symport or translocase driven uptake systems are required for uptake (Olivera, Minambres et al. 1998; Clark, Momany et al. 2002; Ledger, Flores-Aceituno et al. 2009; Wang, Xu et al. 2011). In contrast, the Ibu-2 subclone uptake data presented herein provides preliminary evidence that the product of *ipfF* alone is sufficient to drive PAA uptake via a membrane-associating coenzyme A ligase mediated vectorial acylation mechanism akin to the uptake of fatty acid driven by FadD in *E. coli* (Weimar, DiRusso et al. 2002). To this author's knowledge, a vectorial acylation uptake system for an aromatic acid would be novel.

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CHAPTER 5

CONCLUSIONS

Pharmaceuticals have been detected in bodies of water all over the world, in treated wastewater used for agriculture (Kinney, Furlong et al. 2006; Siemens, Huschek et al. 2008; Xu, Wu et al. 2009) and even in human drinking water (Fent, Weston et al. 2006; Benotti, Trenholm et al. 2008; Huerta-Fontela and Ventura 2008; Corcoran, Winter et al. 2010; Kummerer 2010). The scientific community has seen a recent surge of interest regarding the fate and effects of pharmaceutical compounds released into the environment, prompted not only by the precautionary principle (Ferrari, Mons et al. 2004; Gardiner 2006) but due to widespread detection of such chemicals (summarized by (Santos, Araújo et al. 2010)) and emerging reports of potential (see www.wikipharma.org (Molander, Agerstrand et al. 2009) for a summary of available data) and observed impacts (Oaks, Gilbert et al. 2004).

The prediction of environmental fate of such a diverse category of chemicals is hampered in part, by limited understanding of the way microbes might degrade such chemicals once they enter the environment. It is in response to such a challenge that this dissertation was undertaken. As the most commonly used member of the diverse class of phenylacetate-based non-steroidal anti-inflammatories, ibuprofen (2-(4-isobutylphenyl)propionic acid) was selected so as to provide insight not only into the fate of a common environmental contaminant but also to provide guidance to aid in the prediction of the fate of similar pharmaceuticals. Indeed the results of the scientific inquiries conducted suggest the utilization of a novel metabolic strategy for the

assimilative metabolism of such chemicals. While such work provides valuable insight, the scope of the challenge posed by environmental release of pharmaceutical compounds is great and will require a more attention from the scientific community.

Pharmaceuticals in the Environment: The scope of the challenge to the scientific community

The lack of understanding regarding the issue of pharmaceuticals in the environment can be categorized into several categories. The complex mixture of low concentrations of biologically active compounds presents a quandary for ecotoxicology and human toxicology (Borgert, Quill et al. 2004; Chou 2006; Baas, van Houte et al. 2007; Cedergreen, Christensen et al. 2008; Crofton 2008; Kortenkamp 2008). The toxicological sciences are based upon a foundation of understanding acute toxic effects of individual compounds and have only recently begun to investigate chronic exposure of ecosystems to single chemicals. The subtle chronic effects of mixtures are largely unexplored territory (Groten, Heijne et al. 2004). Ecotoxicology and ecology are tasked with an even more complicated challenge; that of how to quantify the subtle toxicological impact(s) of mixtures on not just single organisms, but on higher levels of biological organization such as populations, communities, and ecosystems.

With respect to the latter problem, the toxicological sciences are faced with the seemingly insurmountable challenge posed by the sheer number of different chemicals present in the pharmaceutical waste stream. Not only is that number already vast, but it

grows ever larger as new products are continually brought into use. The cost of bringing a single pharmaceutical to market has been estimated to be an average of $897 million dollars (Kaitin 2003), of which $466 million is spent on human testing phases and $2-9 million on testing on model organisms (DiMasi, Grabowski et al. 2004). These procedures take years. It is highly doubtful that it will ever be feasible using existing methods to test every compound for subtle and mixture effects. It is in response to such challenges that computational and predictive methods are being increasingly used (Kulkarni, Zhu et al. 2005; Collins, Gray et al. 2008), along with the development of cell-based high through put studies (Inglese, Auld et al. 2006). Large-scale projects by the U.S. EPA, National Institutes of Environmental Health Sciences and National Institute of Health are under way to create comprehensive toxicology databases and focus high-throughput screening efforts, although almost none of these efforts take into account microbial metabolism of these compounds (NIH 2011; Tox21 2011; ToxCast 2011).

If one is presented with several novel chemicals yet have only the capacity to investigate the toxicology of a single compound, one must employ some rational method of deciding which chemical to analyze. While computational tools based on structure activity relationships exist for the prediction of ecotoxicological effects of a given chemical which can help guide such decisions (reviewed in (Netzeva, Pavan et al. 2007; Pavan and Worth 2008; Piparo and Worth 2010; Serafimova, Gatnik et al. 2010) these models are dependent upon thorough scientific data (Scior, Medina-Franco et al. 2009) describing known endpoints or mechanisms of action which calls in to doubt their ability to predict toxicity in the case of novel mechanisms of intoxication or when appropriate data sets are lacking (Greene 2002). An added level of complexity comes from the fact

that the overall impact of a given chemical is not based only upon the chemical itself, but also upon the breakdown products of that chemical. The type and importance of metabolites can vary due to inter-individual differences in human metabolism (Baillie 2007; Uetrecht 2007; Ulrich 2007; Li 2009), human gut metabolism (Doherty and Charman 2002; Li, Wang et al. 2008 ; Thelen and Dressman 2009; van Herwaarden, van Waterschoot et al. 2009) metabolism by environmental microbes (Neuwoehner, Zilberman et al. 2010), and abiotic breakdown products. Science is beginning to develop tools for predicting the breakdown products of a particular pharmaceutical compounds (Kulkarni, Zhu et al. 2005) and make predictions of likely metabolites, but once again, prediction of metabolism relies upon known pathways for similar chemicals. Reliable predictions of a novel compound's metabolism and toxicity are most likely to be accurate only when they are based on experimental data from closely related compounds. Thus we are still left with a daunting task when it comes to hazard identification and risk characterization which underscores the value of the precautionary principle for protecting human and environmental health.

The work presented in this dissertation represents the first investigation into the assimilative bacterial metabolism of an alpha-branched phenylacetic acid with the goal of providing insight that may eventually help inform model development and pathway prediction accuracy

Metabolism of Ibuprofen by Sphingomonas *Ibu-2*

Sphingomonas Ibu-2, which was isolated by enrichment culture from sewage sludge, has the ability to grow using ibuprofen as sole carbon and energy source. Ibu-2 exhibits a slight tendency to degrade the R-enantiomer more quickly than the S, but is capable of growth on either (Figure 2.1). Ibu-2, in an unprecedented fashion, accumulates de-acylated catechols when incubated with a ring-cleavage inhibitor (Murdoch and Hay 2005). The expression of seven open reading frames, *ipfABDEFHI,* proved sufficient to transfer this metabolic activity upon an *E. coli* host. Uptake assays performed using an Ibu-2 chromosomal library clone capable of performing this deacylating reaction suggests that Ibu-2 accumulates ibuprofen intracellularly through the action of a coenzyme A ligase enzyme encoded by *ipfF* and powered by ATP. Coenzyme A ligation encourages uptake on two general principles. Firstly, the coenzyme group is large and hydrophilic, thus trapping ibuprofen inside the cell. In the absence of ligation, ibuprofen is able to cross cellular membranes in both directions, resulting in no net intracellular accumulation. However, by chemically altering it, ibuprofen is effectively taken out of the thermodynamic equation therefore encouraging continued influx. The combination of intracellular trapping and continued influx down a concentration gradient through a ligative process is termed vectorial acylation and has also been observed in the metabolism of fatty acids by bacteria and eukaryotes (Weimar, DiRusso et al. 2002; Black and DiRusso 2003; Zou, F. et al. 2003).

Ibuprofen coenzyme A (ipfCoA) is the likely target of further metabolism by a dioxygenase system in Ibu-2. This conclusion is based on the requirement of functional

copies of *ipfA* and *ipfB* (genes share homology with the two primary components of aromatic dioxygenase systems) in pFOS3G7 for isobutylcatehcol production and upon the observation that subcloned *ipfABDEF* is not capable of efficient metabolic activity without the co-expression of *ipfHI,* a ferredoxin reductase and ferredoxin respectively (Figures 3.9 - 3.11). The presumed 1,2 dearomatized diol that results from IpfAB-mediated transformation of ibuprofen CoA is predicted to be the substrate that leads to isobutylcatechol production via IpfDE.

The predicted amino acid sequence of *ipfD* shares homology with genes encoding acyl-transferases that are members of the sterol carrier protein X (SCPx) family and which are known to catalyze the removal of an acyl-CoA group. Despite this family being numerous and diverse, it is poorly characterized. In fact, the limited experimental exidence presented in chapter 3, which shows that expression of *ipfDE* is essential for isobutyl catechol production from ibuprofen, makes it only the third bacterial putative SCPx protein that has been characterized at the reaction level. Via an unknown mechanism, ibuprofen CoA diol is rearomatized to isobutylcatechol during or after the deacylation reaction. The role played by the domain of unknown function 35 (DUF35) protein IpfE in this process is unknown, but it likely associates with the acid side chain and is necessary for the activity of IpfD given the fact that many DUF35 genes are found next to a SCPx gene in sequenced genomes. The likely involvement of an SCPx thiolase and DUF35 protein in the direct conversion of a phenylacetic acid derivative directly to a de-acylated catechol was not forseen and is without precedent. This novel reaction mechanism may prove relevant for the prediction of possible environmental breakdown products of other alpha-branched phenyalcetic acids such as ketoprofen and naproxen

(figure 1.1). While the recently characterized *paa* bacterial pathway might prove more useful for the breakdown prediction for non-alpha-branched NSAIDs with a phenylacetic acid core such as diclofenac, etodolac, indomethacin, sulindac, and tolmetin, it is at this point unknown how wide spread *ipf*-like pathways might be and what their specifity might be.

Points of Uncertainty in the Ibu-2 Ibuprofen Metabolism Model

The vectoral acylation mechanism conclusion was based partially upon the observation that adding the metabolic poisons potassium cyanide or dimethylphenol to *E. coli* epi300 pFOS3G7 inhibited uptake, indicating the presence of an active transport system. Using the same poisons on *E. coli* epi300 pFOS3G7 with a knocked-out *ipfF* did not impact uptake to any degree; this is a critical piece of data indicating that IpfF is the only source of active transport amongst the DNA expressed on pFOS3G7. It may be pointed out that cyanide and 2,4-DNP poison both affect the chemiosmotic gradient and as a consequence ATP production and therefore the loss of uptake upon poisoning could result in the disruption of gradient-driven transport by a major facilitator porin transport system. A thorough approach would have utilized an ATP synthase inhibitor such as venturicidin (Hong and Pedersen 2008) while leaving the chemiosmotic gradient undisrupted. However, the *E. coli* epi300 pFOS3G7Tn:*ipfF* data obviates the need for this by demonstrating that no other active transport system is active in *E. coli* epi300 pFOS3G7.

While IpfF is sufficient for uptake in *E. coli*, it may not be so in *Sphingomonas*. It is possible that transport in Ibu-2 also involves a FadL-like translocase such as has been proven to enhance the uptake of fatty acids (Ferguson and Deisenhofer 2002; van den Berg, Black et al. 2004) and of toluene (Wang, Rawlings et al. 1995; Kahng, Byrne et al. 2000).

While *ipfABDEFHI* were shown to be sufficient for metabolic activity, conclusions regarding the activity of the putative enzymes IpfAB and IpfDE are based on indirect experimental data . Each of the associated genes has been demonstrated to be necessary for ibuprofen metabolism. The hypothesis that IpfAB acts upon the coenzyme A CoA-ligate that is the product of IpfF is supported by the statistically significant greater loss of phenylacetyl-CoA in *E. coli* pGEM:*ipfAB* cell-free extracts. However, the difference between the clone and the control was modest (though statistically significant) and no reaction products could be identified via chromatographic methods. Additionally, the requirement of subcloned *ipfABDEF* on the co-expressed electron transport chain *ipfHI* (Figure 3.10 – 3.11) offers strong but indirect evidence for the involvement of *ipfAB*.

The activity of IpfDE is largely speculative and based primarily on the inference that IpfDE is likely responsible for metabolizing dioxygenated ibuprofen-CoA to isobutylcatechol. This inference is supported by catalytic activity described for orthologs of IpfD; SCPx acyl-transferase proteins which deacylate alpha-branched 3-oxo CoA-ligated fatty acids in both eukaryotes (Wanders, Denis et al. 1997; Verhoeven and Jakobs 2001; Westin, Hunt et al. 2007) and bacteria (Leuthner and Heider 2000; Kube, Heider et al. 2004; Kuhner, Wohlbrand et al. 2005). The inferred involvement of IpfE in the

process is based upon the fact that ibuprofen metabolism is dependent upon its presence and that an IpfE-like DUF35 protein and an SCPx thiolase have been shown to be intimately associated in the case of benzylsuccinate metabolic proteins BbsB and BbsC (Leuthner and Heider 2000; Kube, Heider et al. 2004; Kuhner, Wohlbrand et al. 2005).

Environmental Relevance of the Ibu-2 Ibuprofen Pathway

A number of questions can be raised regarding the relevance of this pathway. Firstly, how common is this pathway? Is it a common paradigm for dealing with ibuprofen and similar chemicals or is it an anomaly? Additionally, it is not at all clear that this is the mechanism whereby ibuprofen would be metabolized in the environment where it is found at much lower concentrations than were used in this work.

To approach the question of whether or not ibuprofen is degraded via the Ibu-2 type pathway by organisms that have only been exposed to low concentrations, active sewage sludge was spiked with ibuprofen and ring-cleavage inhibitor and metabolites were analyzed within only a few hours, presumably before any genetic acclimatization could take place (Appendix 4). While many structural variations of hydroxyibuprofen and carboxyibuprofen were detected, isobutylcatechol was not detected, suggesting that the resident microbial community was not expressing an Ibu-2 type pathway. This preliminary piece of data suggests that the isobutylcatechol pathway may not be relevant in the environment in a strict sense although catechols could have sorbed or reacted with the abundant organic matter present in the sewage sludge and therefore went undetected. This issue was addressed directly in a pilot project that applied a bacterial fractionation of

environmental samples in order to remove the presumptive solid phase catechol sink (outlined in Appendix 4). The bacterial fractions did indeed produce traces of acid labile yellow color consistent with meta cleavage product when spiked with the ibuprofen analog *m*-tolylacetica acid, suggesting that such pathways might indeed be present at appreciable levels in the environment.

The apparent discrepancy regarding the fate of ibuprofen at high versus low concentrations is one of far-reaching relevance. It is not entirely clear what happens to low-concentration organic chemicals in the environment. Martin Alexander's laboratory (Rubin, Subba-Rao et al. 1982; Schmidt, Alexander et al. 1985) approached this topic and presented evidence that an entirely different set of organisms acts upon organics at low versus high concentrations. Low concentrations of organics were mineralized via unknown and apparently non-assimilative mechanisms, which is reminiscent of the seemingly non-specific oxidation of ibuprofen by the biological activity present in sewage sludge. The mineralization reported by Alexander and coworkers obviously excludes the assimilative types of metabolism that are present in microorganisms isolated by the enrichment techniques used in this study. The possible mechanisms of low concentration metabolism could be but are not limited to:

1. Fungal extracelluar lignin-degrading enzymes such as fungal laccases; Laccases, which are present in prokaryotes, fungi, and plants, have a wide variety of functions (Mayer and Staples 2002; Claus 2004). Fungi are known to secrete laccases for the putative purpose of catalyzing the oxidative degradation of the lignin polymers into smaller molecules that can be been absorbed (Claus 2003). Because lignin is an

extremely structurally complex compound, a wide variety of laccases with varying specificity are secreted. While it has never been directly investigated, it seems likely that this constant presence of laccases in biological slurries is likely to have the capacity to non-specifically oxidize many organic xenobiotics. Indeed, it has been demonstrated that laccases can oxidize a wide variety of phenolics and aromatic anilines (Claus 2003) and that they oxidize the carbons of a xenobiotics in a fairly random manner (Ullrich and Hofrichter 2007) such as was observed during incubations of ibuprofen in sewage sludge.

2. Cometabolism, or the biotransformation of xenobiotics by metabolic processes designed for another purpose but with loose specifity; cometabolic processes have been found to be significant in the fate of MTBE (Fiorenza and Rifai 2003), chlorinated ethylenes (Arp, Yeager et al. 2001), and polycyclic aromatic hydrocarbons (Prak and Pritchard 2001). Such processes may be at work in metabolizing very low concentrations of xenobiotics when the enzymes in question have been induced by other chemicals for which they are specific.

3. Organisms that are poorly represented in enrichment and culturing processeses; it is widely acknowledged that the majority of prokaryotes present in the environment cannot be recovered using traditional culturing techniques (Amann, Ludwig et al. 1995). This means that any given bacterium performing a relevant metabolic process in the environment is likely to be uncultivable.

Future Work

The relevance of this work straddles two realms, environmental toxicology and microbial metabolism. From the microbiological perspective, this work's contribution lies in providing preliminary evidence for new types of enzymes and metabolic pathways that may provide a deeper understanding of bacterial metabolic potential in general. From an environmental perspective, this work was performed with the hope that information would be gained that would aid in the prediction of the environmental fate of similar xenobiotics. For example, the metabolic pathways responsible for the elimination of the phenylacetic acid based pharmaceuticals diclofenac, naproxen, ketoprofen, and benzafibrate are all currently poorly understood (Quintana, Weiss et al. 2005; Kosjek, Heath et al. 2009). Degradation of each of these chemicals in environmental systems has been observed, but the intermediates have been scarcely described. The 1,2 dioxygenase, SCPx, DUF35 deacylating pathway offers novel hypothetical metabolites to search for in each case.

Future Work: Environmental

First of all it would be informative to know whether or not *ipf*-type genes are found with any frequency in the environment. While it would be informative to examine environmental samples using degenerate primers or probes for bacterial SCPx genes, this family of genes is very divergent; sequence alignment does not reveal any suitably conserved targets for analysis. In order begin to address the general question of whether such a pathway is present at appreciable levels in the environment, a pilot experiment

was conducted in which ibuprofen and *m*-tolylacetic acid were spiked into the bacterial fraction of Cayuga lake water (described in Appendix 4). A yellow coloration consistent with the presence of meta-cleavage product of methylcatechol was present though the absorbance peak was somewhat broad and inconclusive. A GC/MS analysis of such samples might reveal the presence of the *meta*-cleavage semi-muconic acid metabolite.

The presence and unique qualities of the *ipf* pathway are puzzling for a variety of reasons. For instance, why is this pathway fundamentally different from the widely distributed *paa* pathway which degrades phenylacetatic acid and phenylalanine via an epoxide intermediate? It is perhaps informative that Ibu-2 grows using the same *ipf* pathway on tolylacetic acids but is unable to grow on phenylacetic acid. This suggests that the *ipf* pathway is suited to the metabolism of methylated phenylacetic acids as opposed to unsubstituted phenylacetic acid. It would be informative to investigate whether the *paa* pathway is capable of activity towards tolylacetic acids. An inability of the epoxide-mediated *paa* metabolic strategy to act upon tolylacetic acids would take a step towards explaining the presence of the unique *ipf* pathway. However, it remains puzzling that while the *ipf* pathway strategy is apparently very well-suited towards the metabolism of phenylacetic acid to catechol and subsequent ring cleavage products, such a strategy has not been reported in the literature for the metabolism of such a ubiquitous and well-studied chemical.

In this and other studies, several varieties of hydroxylated ibuprofen have been identified in spiked sewage sludge or environmental samples. In order to approach the role of fungal laccases in the production of the hydroxylated metabolites that have been identified, the following research strategies could be utilized. Unfortunately, laccase

inhibition is out of the question due to the fact that the only true laccase inhibitor is azide, which also has broad biocidal properties (Johannes and Majcherczyk 2000). Alternatively, it would be very informative to use pure fungal strains or to clone laccases and specifically examine their capacity to oxidize ibuprofen and other xenobiotics.

Radiolabelled ibuprofen could be used to trace the fate of ibuprofen in environmental samples. Unfortunately only ibuprofen that is labeled on the carboxy carbon is currently available commercially. This is a very limiting factor due to the observation that Ibu-2 removes the carboxy atom early in the metabolic process. If ring labeled ibuprofen could be synthesized, much more could be learned. For example, trace amounts of labeled ibuprofen could be added to sewage sludge, the carbon dioxide released during mineralization could then trapped and quantified or HPLC and GC/MS could be used to detect and identify radiolabelled metabolites. Further analysis could be performed on the biological fraction to detect any assimilated radioactivity. For example, a radioactive variation of Stable Isotope Probing (SIP) (Madsen 2006) which uses cesium chloride gradients to separate DNA based on the mass of the carbon isotope it contains could be used to obtain a phylogenetic snap-shot of the organisms that are assimilating labeled ibuprofen.

Future Work: Microbiological

From a microbiological perspective, the most intriguing aspect of the system by far is the apparently novel removal of the acyl-CoA group and associated re-aromatization of the ring. No such chemical reaction has been reported in the literature.

The pathway was successfully reconstructed using subcloned *ipfABDEFHI.* Presently there is only indirect evidence that IpfABHI dioxygenates CoA ligated substrate. Directly identifying the oxygenated metabolite of *ipfABFHI* would both remove uncertainty on this point and also begin to offer insight into the mechanism of *ipfDE.* The SCP-x thiolase and DUF35 *ipfDE* are the most exciting aspect of this system given the ubiquity of these types of genes and the near complete lack of insight into the functions of their products. The pFOS3G7ΔD mutant should be adequate for production of the dioxygenase metabolite. The fosmid, when upregulated, is at a high copy number. However, the activity of the pFOS3G7 system is not remarkably high. It is possible that cloning *ipfABFHI* into a high copy number vector with a strong *E. coli* promoter will prove more fruitful. An *ipfABF* construct in pGEMt-easy, which is a high copy number vector with a strong *lac* promoter, has already been constructed. pBBR1mcs could be used to express *ipfHI.* Alternatively, the *ipfABF* cassette could be combined with *ipfHI* on a single vector using strand overlap extension.

In order to definitively identify the dioxygenated metabolite, NMR should be employed. GC/MS identification of a diol is possible but challenging, requiring the use of a butylboronic acid derivitization. Even then, GC/MS does not provide definite identification of a chemical in the absence of a standard. In order to perform NMR, substrate completely labeled with carbon-13 must be obtained. [U-13C]phenylacetate is not commercially available, but a farily simple method for it's production is in publication (Ismail, Mohamed et al. 2003) by which phenylalanine is deaminated using commercially available L-amino acid oxidase. The reaction is then acidified and extracted with ethyl acetate in order to purify the reaction product.

Teufel *et al.* (Teufel, Mascaraque et al. 2010) produced 13C-labelled intermediates in bacterial phenylacetate production by first developing methods by which to purify the proteins. Maltose binding protein tagging was used instead of traditional histidine-tags in most cases for unspecified reasons. Developing methods by which to purify IpfABFHI will be required as a first step. Teufel *et al.* column-purified overexpressed proteins and then exposed them to substrate with all necessary cofactors, which in the case of IpfABFHI will likely require a pool of ATP, CoA, and NADH. Alternatively, the reaction could be split into the two substituent steps, i.e. separate reactions and product purifications for first the IpfF coenzyme A ligation and then the IpfABHI dioxygenation. The reaction products can be purified by HPLC. Teufel *et al.* also included radioactive 14C-labelled substrate as a tracer to aid in this step. The 14C substrate would also have to be prepared from 14C phenylalanine using the amino acid oxidase method described above. Regarding product stability, Teufel *et al.* found that most intermediates in the *paa* pathway had half-lives in methanol ranging from 2.5 to 6 hours. Half-lives in water were very low. Following product purification, the samples should be dried, resuspended in deuterated water and subjected to NMR analysis.

Further characterization of *ipfD* and *ipfE* will prove challenging given that these types of gene products have never been characterized at the reaction level. I have constructed histidine-tagged *ipfD* and *ipfE* on protein overexpression vectors and they might be prove useful for further characterizations by allowing for the purification of the protein products. Unfortunately, the likely instability of the hypothetical diol metabolite will make generation of a stable chemical stock by an *ipfABFHI* construct challenging if not impossible. If the reaction product of IpfABFHI can be identified by NMR as

described above, the reaction product can be exposed to purified IpfD and/or IpfE in order to verify that it is indeed the substrate for the subsequent and final reaction.

Chapter 4 described the investigations into the transport mechanisms employed by the *ipf* system. The results suggested that the predicted protein product of *ipfF* was responsible for uptake of the substrate via a CoA-mediated vectorial ligation mechanism. However, these experiments were performed exclusively in an *E. coli* host using radiolabelled phenylacetic acid. Ibu-2 proved difficult to work with due to the fact that it clogged the filter membranes and phenylacetic acid was used due to the inexpensiveness of radiolabelled phenylacetic acid versus ibuprofen. While the results were consistent with the known mechanisms for fatty acid uptake systems, they were at odds with other described systems for aromatic acid uptake, including those for phenylacetic acid. The bulk of phenylacetic acid uptake systems involve specific porin-type active-transport systems. While IpfF may have been sufficient to mediate uptake in the *E. coli* host, it is possible that other transport proteins are involved or perhaps even required in *Sphingomonas* Ibu-2. In order to study uptake in Ibu-2, a different approach would have to be taken. Either an efficient way to remove the extracellular matrix from Ibu-2 would have to be developed or an uptake assay that does not involve filter membranes would have to be utilized. If an alternative uptake protocol could be developed, the question of the sufficiency of IpfF to drive uptake could be addressed by a targeted knockout of *ipfF* in Ibu-2 and measuring uptake. Initial attempts to generate genetic knockouts in Ibu-2 using targeted double recombination or random transposon based approaches proved untenable (Appendix 4). Alternatively, *ipfF* could be cloned into a more closely related

species, one that is more amenable to filter-based uptake assays, and the uptake studied therein.

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APPENDIX 1

A NOVEL MECHANISM FOR THE BIODEGRADATION OF IBUPROFEN

Preface

The following manuscript was prepared in 2002 and submitted to Environmental Science and Technology. It was accepted on the condition that certain experiments be elaborated upon. Attempts to revive the organism that was supposedly isolated and identified as a *Variovorax* sp. Ibu-1 were unsuccessful. It seemed the case that Ibu-1 was not a pure culture and in fact had been, at some point in the course of regular maintenance of the organism, heavily contaminated. Whether the dihyroxy- and trihydroxyibuprofen detected in culture media were actually utilized in a catabolic pathway remains uncertain. Subsequent attempts to isolate an organism utilizing a similar pathway, distinct from the isobutylcatechol pathway utilized by Ibu-2, proved fruitless.

Abstract

A bacterium was isolated from sewage sludge that has the ability to use ibuprofen as its sole carbon and energy source. 16S rRNA gene sequence analysis revealed 98% homology to species of the *Variovorax* genus.

When grown on ibuprofen it accumulated a transient yellow intermediate that disappeared upon acidification, a trait consistent with *meta* ring-fission metabolites. GC/MS analysis of the derivatized culture supernatant yielded two spectra consistent with trihydroxyibuprofen, bearing all three hydroxyl groups on the aromatic ring. However, these metabolites were only detected when 3-fluorocatechol, a *meta* ring-fission inhibitor, was added to Ibu-1 cultures and the supernatant was then derivatized with aqueous acetic anhydride and diazomethane. These findings suggest the possibility of ibuprofen metabolism proceeding via a *meta* ring-fission pathway. Identical spectra, consistent with these putative ring-hydroxylated trihydroxyibuprofen metabolites, were also obtained from ibuprofen-spiked sewage sludge, but only when it was poisoned with 3-fluorocatechol and derivatized. This demonstrates the biological potential for ibuprofen degradation via previously unidentified intermediates. The presence of the same metabolites in both spiked sewage sludge and culture supernatants suggests that a ring-cleavage pathway for the degradation of ibuprofen may have environmental relevance.

Introduction

Ibuprofen (2-(4-isobutylphenyl-propionic acid)) is a non-steroidal anti-inflammatory drug (NSAID) used for its analgesic, antipyretic, and anti-inflammatory properties. Two decades ago, it was labeled as being "inherently biodegradable"

(Richardson and Bowron 1985). In the decades since, ibuprofen has become the third most highly consumed drug in the world (Buser, Poiger et al. 1999). Given that 8.9% - 14% of the administered dose is excreted unmodified or as the easily hydrolysed glucuronide conjugate (Lee, Williams et al. 1985; Rudy, Knight et al. 1991; D'Ascenzo 2003), a large amount of ibuprofen has the potential to enter the environment.

Environmental concentrations of ibuprofen have been found to range from low ppt (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Farre, Ferrer et al. 2001; Winkler, Lawrence et al. 2001; Kolpin, Furlong et al. 2002) to low ppb levels (Buser, Poiger et al. 1999; Farre, Ferrer et al. 2001). However, these studies analyzed only the ibuprofen in aqueous solution. With a log K_{ow} of 3.5 (Stuer-Lauridsen, Birkved et al. 2000), ibuprofen would be predicted to partition predominantly to sediment.

Despite the large environmental load of ibuprofen, little is known regarding how ibuprofen is degraded by bacteria in the environment. No organisms have been reported to grow on ibuprofen in pure culture, although Chen and Rosazza (Chen and Rosazza 1994) reported that a *Nocardia* species could reduce the carboxylic acid moiety to an alcohol and then acetylate it. In laboratory reactor experiments, Winkler *et al.* (Winkler, Lawrence et al. 2001) reported an increase in the concentration of ibuprofen with a hydroxyl- or carboxyl-group on a side-chain concomitant with the decrease in concentration of ibuprofen. In addition to these metabolites, Zwiener *et.al*. (Zwiener, Seeger et al. 2002) also reported 4-carboxyhydratropic acid as a degradation product of ibuprofen. However, these metabolites did not account for all ibuprofen degradation, representing only 10% of total ibuprofen disappearance in one case (Zwiener, Seeger et al. 2002).

Like ibuprofen, many other NSAIDs, including naproxen, ketoprofen, and diclofenac, are aliphatic-substituted phenylacetic acids. There is extensive literature describing the bacterial metabolism of hydroxyphenylacetic acids (Sparnins and Chapman 1976; Blakley 1977; van den Tweel, Smits et al. 1988) and some recent work regarding phenylacetic acid, which proceeds via a phenylacetyl CoA intermediate (Ferrandez, Minambres et al. 1998; Olivera, Minambres et al. 1998). However, there has been almost no work describing the biodegradation of aliphatic-substituted phenylacetic acids, including ibuprofen. As perhaps the most ubiquitous member of these two overlapping classes of compounds, ibuprofen serves as a useful model for how these compounds may be metabolized.

With the exception of unsubstituted phenylacetic acids, the literature regarding the aerobic metabolism of aromatic compounds by bacteria reveals a near-constant paradigm. The aromatic ring is dioxygenated on vicinal carbons and dehydrogenated to form a catechol, followed then by the insertion of molecular oxygen to accomplish ring opening (Butler and Mason 1997). *para*-dioxygenation is more rare, but is also a sufficient prerequisite for ring-opening (van den Tweel, Smits et al. 1988). If bacteria are to completely metabolize ibuprofen under aerobic conditions, they might do it by dioxygenating the ring.

While catechols seem to be a common intermediate in the aerobic metabolism of aromatic compounds, they are ephemeral, being transformed rapidly by both enzymatic and non-enzymatic mechanisms (Schweigert 2001). For these reasons, they are rarely detected in the environment. As catechols are known to be toxic (Schweigert 2001), most organisms capable of degrading aromatic compounds do not normally accumulate

catechols without either prior genetic manipulation or the use of enzyme inhibitors. Given the uncertainty regarding the fate of most of the ibuprofen entering the environment, we hypothesized that ibuprofen may be metabolized to intermediates that are not readily detected using standard sample preparation techniques, and have therefore previously gone undetected. Specifically we hypothesized that ibuprofen might be degraded via initial ring dioxygenation followed by *meta*-cleavage. In order to test this hypothesis we characterized ibuprofen metabolites from an environmental bacterium named Ibu-1, which is capable of using ibuprofen as a sole carbon and energy source. We then examined ibuprofen-supplemented sewage sludge for the presence of the same metabolites.

Materials and Methods

Materials. All chemicals were purchased from Acros (Morris Plains, NJ). Sewage sludge was collected from the City of Ithaca, NY sewage treatment plant.

Enrichment of Sewage Sludge. Sewage sludge was enriched with S-ibuprofen according to standard protocols (Krieg 1981). After five serial transfers, the final enrichment was streaked onto a solid mineral salts medium (MSM) containing 500 mg/L S-ibuprofen. MSM was composed of 1 mM $MgSO_4$, 10 mM K_2HPO_4, 3 mM NaH_2PO_4, 10 mM $(NH_4)_2SO_4$, 10 μM $Fe(NO_3)_3$, and 100 μM $Ca(NO_3)_2$ (McCullar 1994). A single colony was isolated and subjected to further analysis.

Species Identification. A portion of the 16S ribosomal rRNA gene of Ibu-1 was PCR amplified using primers 1055F (ATGGCTGTCGTCAGCT) (Ferris 1996) and 1492R (TACGGYTACCTTGTTACGACTT) (Lane 1991). The resulting product was

then ligated into pGEMt-easy (Promega, Madison, WI) and electroporated into *Escherichia coli* DH5α. The insert contained within a single colony was amplified using T7/M13 primers and sequenced. The sequence was then analyzed via BLAST (Altschul 1990), an online database and search engine which identifies analagous DNA sequences.

Growth Conditions of Ibu-1. The isolate was grown in liquid MSM and containing 500 mg/L S-ibuprofen and supplemented with 0.2% Luria-Bertani broth (LB) to speed growth. Growth was monitored by measuring absorbance at 600 nm using a 96 well μQuant spectrophotometer from BioTek instruments (Winooski, VT).

Monitoring Ibuprofen Concentrations via HPLC. 1 ml of Ibu-1 culture was centrifuged at 15,800 x g for 2 minutes. The supernatant was then passed through a 0.2 μm filter. Results from the HPLC analysis of samples and a standard curve were used to determine the ibuprofen concentration in the supernatant. The eluent was 70% methanol and 30% 40 mM acetic acid. The sample was pumped at a rate of 1 ml/min using a Waters Model 590 pump. The column was a Varian Microsorb-MV C18 column, 250 mm by 4.6 mm. Under these conditions, ibuprofen elutes at approximately 15 minutes. Samples were injected by a Shimadzu SIL-10AD AP autoinjector. Detection was accomplished with a Shimadzu SPD-10A VP UV-Vis detector measuring absorbance at 220 nm.

GC/MS Analysis of *Variovorax* Ibu-1 Culture Supernatant. Two 100 ml ibu-1 cultures were grown on 500 mg/L S-ibuprofen until late exponential phase. To one culture, 3-fluorocatechol (3FC) was added to a final concentration of 50 mg/L. After thirty minutes at room temperature, the cultures were centrifuged and the supernatants pulled through a 0.2 μm filter. Aqueous acetylation was performed by adding potassium

carbonate and acetic anhydride to the supernatant to final concentrations of 1.5% and 0.5% respectively (Mars 1997). After thirty minutes, the samples were acidified to pH 3, extracted with ethylacetate, and dried over a sodium sulfate column. The extracts were then methylated with diazomethane. Diazomethane was generated in a 40 ml EPA vial by combining 5 ml ethyl ether with 3–5 g of N-nitroso-N-methylurea, a single pellet of sodium hydroxide, and 0.1–0.2 ml of water. The ethyl ether containing diazomethane was dried over a sodium sulfate column, and then added to the ethylacetate extracts. After thirty minutes at room temperature, the samples were evaporated to a minimal volume under a nitrogen stream and separated using an HP 6890 GC equipped with an HP-5MS column (5% phenyl methyl siloxane 30 m x 0.25 mm, 0.25 μm film thickness) with helium as the carrier gas at a flow rate of 1 mL/min. The injector temperature was 250°C. The initial oven temperature of 40°C was held for 1 min, then ramped at a rate of 10°C/min. to 250°C. The temperature was held at 250°C for 7 min and then ramped up at 30°C/min until 300°C. The detector was an HP 5973 MSD with quadrapole and source settings of 150 ºC and 230 ºC respectively.

GC/MS Analysis of Ibuprofen Supplemented Sewage Sludge. 500 mg/L R/S ibuprofen was added to 1 L of sewage sludge. Ibuprofen concentration was monitored via HPLC as described above. After one week the ibuprofen concentration had dropped to approximately 50% of original level and the sample was split in two. To one half, 3-fluorocatechol was added to a final concentration of 1000 mg/L. After thirty minutes, the sludge samples were centrifuged. The supernatant was then filtered sequentially over a 4 μm filter followed by a 0.4 μm filter. The samples were then extracted, derivatized, and analyzed via GC/MS as described above.

Results and Discussion

Isolation of a Bacterium capable of growth on Ibuprofen. Ibu-1 was isolated from an ibuprofen enrichment culture that had the ability to use S-ibuprofen as a sole carbon and energy source. Growth of Ibu-1 correlated directly with the disappearance of ibuprofen (Figure A1.1).

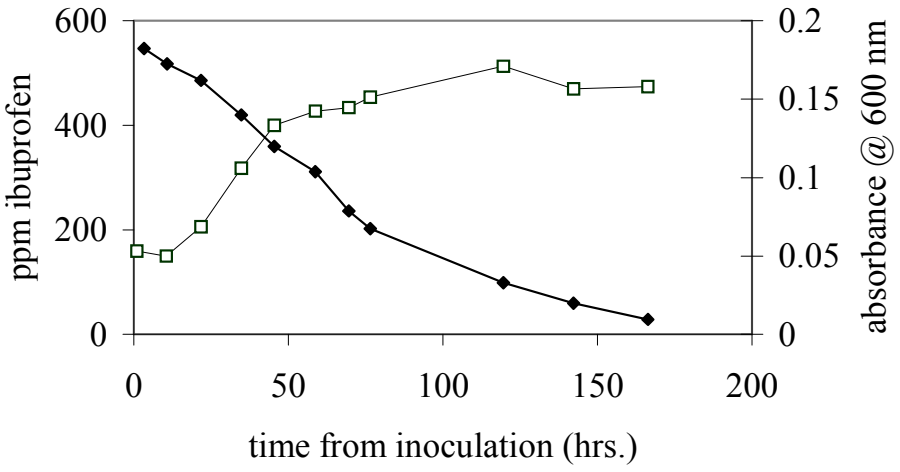

Fig. A1.1. Growth of Ibu-1 on ibuprofen as measured by increase in Ibu-1 culture density via spectrophotometry at 600nm (u). Concomittant loss of ibuprofen during growth as measured via HPLC (p).

Ibu-1 entered exponential growth phase 10 to 20 hours after inoculation into 500 mg/L ibuprofen in MSM (fig. A1.1). Stationary phase was reached after approximately 75 hours. By this time, approximately 40% of the initial ibuprofen had been metabolized. When Ibu-1 was grown in ibuprofen/MSM liquid, a yellow color appeared in the supernatant. This yellow color disappeared upon acidification, and reappeared upon

neutralization. This pH-dependent yellow color is indicative of typical *meta*-cleavage products (McCullar 1994).

BLAST (Altschul 1990) analysis of the 16S ribosomal rRNA gene sequence revealed Ibu-1 to be a β-proteobacterium, having 98% homology with species of the *Variovorax* genus. This genus is represented by a large number of environmental isolates that have been best characterized with respect to their ability to metabolize 2,4-dichlorophenoxyacetic acid (2,4-D) (Giovanni, Neilson et al. 1996; Vallaeys, Albino et al. 1998; Smejkal, Vallaeys et al. 2001). In addition to utilizing 2,4-D, some members of this genus such as *V. paradoxus* have also been reported to be capable of growth on benzoate (Maskow and Babel 2001). However, degradation of both of these compounds by *Variovorax* has been reported to take place via *ortho*-cleavage.

GC/MS of *Variovorax* Ibu-1 Culture Supernatant. Ibu-1 cultures were exposed to 3FC, which inhibits *meta*-cleavage enzymes (Bartels 1984), and resulted in the accumulation of ibuprofen catechols (Mars 1997). Underivatized catechols are not usually amenable to analysis via gas chromatography. To overcome this problem the supernatant was derivatized with aqueous acetic anhydride, a procedure that adds acetyl groups specifically to aromatic hydroxyl-groups (Fujimoto 1997). Thus, derviatization not only permitted good analyte separation but, when coupled with mass selective detection, gave diagnostic mass spectra that allowed for the identification of ibuprofen metabolites substituted with aromatic hydroxyl groups. Two mass spectra consistent with ring-hydroxylated ibuprofen were detected in 3-fluorocatechol-treated Ibu-1 supernatant that had been derivatized with acetic anhydride (Fig. A1.2).

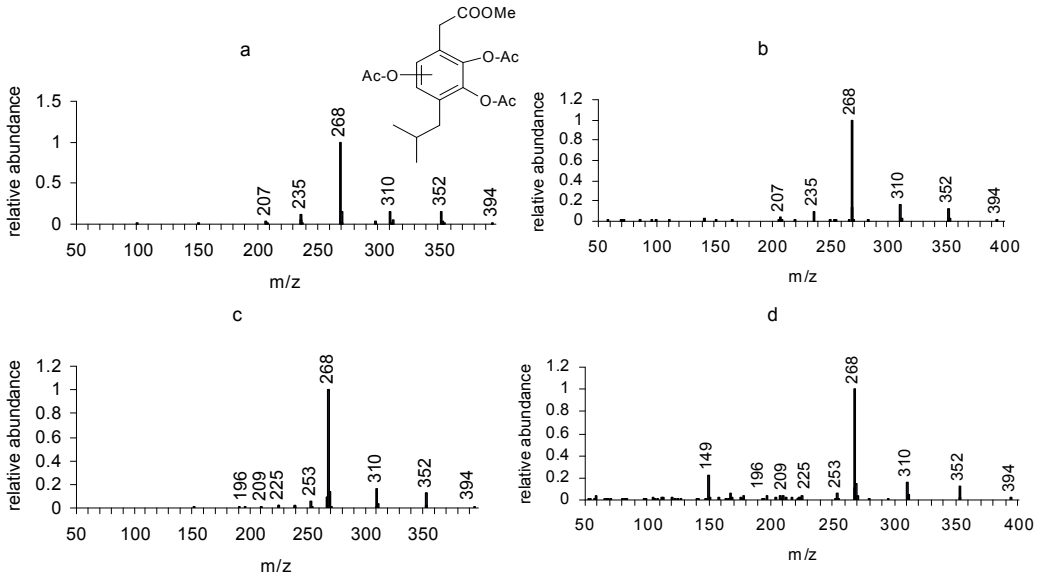

Fig. A1.2. Spectra of putative metabolites identified via GC-MS analyses. Figures (a) and (b) (Rt 22.7 min) from Ibu-1 culture supernatant and sludge respectively. Figures (c) and (d) (Rt 27.4 min) from Ibu-1 culture supernatant and sludge respectively. These spectra were only detected in extracts after 3-fluorocatechol poisoning followed by aqueous acetylation. "Ac" is an acetyl group, "Me" is a methyl group.

These spectra were not detected in the absence of either 3-fluorocatechol or derivatization. Both spectra contained a parent ion at 394 and were consistent with methylated triacetylated trihydroxyibuprofen. Three separate losses of 42 resulted in peaks at 352, 310, and 268. A loss of 42 is diagnostic of the fragmentation of an acetyl ester. The aqueous acetylation step employed during derivatization has been shown to acetylate only aromatic hydroxyls (Fujimoto 1997). Two such spectra appeared, one at 22.7 minutes and the other at 27.4 minutes, presumably representing the two possible isomers of ring hydroxylated trihydroxyibuprofen.

Dihydroxyibuprofen was the intermediate that was expected based upon analogy to metabolic pathways employed to degrade similar compounds, such as ρ-cumate (Defrank and Ribbons 1976; Defrank and Ribbons 1977; Eaton 1996; Eaton 1997). ρ-

Cumate (4-isopropylbenzoate) is similar to ibuprofen in that it too is an aromatic acid with a branched aliphatic group in the *para* position. It is the only such compound whose degradation pathway has been described. ρ-Cumate is dioxygenated at the 2,3 position by *Pseudomonas putida* F1 carrying the *cmt* operon. It is then subsequently dehydrogenated to yield 2,3-dihydroxy-4-isopropylbenzoate. This compound is then cleaved via dioxygenation across the 3,4-bond to yield a diagnostic pH-dependent yellow *meta*-cleavage product.

Although not a canonical intermediate of aromatic metabolism, the presence of trihydroxyibuprofen could be explained two ways. First, it may be a dead-end metabolite produced only in the presence of 3-fluorocatechol. Under normal growth conditions, i.e. when the ring cleavage inhibitor was not present, these poly-hydroxy metabolites were not observed. When ring-cleavage is inhibited, the putative ibuprofen hydroxylating enzyme(s) may fortuitously oxygenate dihydroxyibuprofen, producing trihydroxyibuprofen . We have observed similar nonspecific oxygenase activity when the same techniques were employed to inhibit ring cleavage of phenol metabolites by *Pseudomonas* sp. CF600 (data not shown). Alternatively, trihydroxyibuprofen may be the true ring-cleavage substrate. Although there is precedent for the degradation of trihydroxybenzenes via ring-cleavage (Haigler 1999), established theory in the field of aromatic metabolism holds that dihydroxylation sufficiently activates the phenyl ring so as to permit ring-cleavage (Harayama and Timmis 1989). As oxygenation is an energetically expensive reaction, it seems unlikely that an organism would waste energy by performing an unnecessary oxygenation unless this somehow facilitated the further

degradation of the ring cleavage product. Additional analysis is needed before the nature of the actual substrate for ring cleavage can be confirmed.

As the vast majority of ring-cleavage pathways involve catecholic intermediates, such a compound is most likely to be a substrate for ring-cleavage. This is supported by the observation that catecholic metabolites were not detected unless the system was poisoned with 3FC, a known catechol dioxygenase inhibitior. Although a yellow metabolite accumulated in culture supernatant which had the characteristics of a ring-cleavage product, GC-MS analysis of methylated extracts failed to yield spectra consistent with a ring fission product of an ibuprofen catechol. This is not surprising however, as *meta*-ring cleavage products have been reported to be recalcitrant to isolation and analysis (Schweigert 2001).

GC/MS of Activated Sludge Supplemented with Ibuprofen. After an initial lag phase of approximately 5 days, the ibuprofen concentration began to drop. Seven days after the ibuprofen addition, approximately 50% of the original 500 mg/L had been degraded. At this point, the liter of sludge was split in half, one half being poisoned with 1000 mg/L 3-fluorocatechol. Analysis of derivatized products from the sludge supernatant via GC/MS revealed spectra identical in all major aspects to those putatively identified as trihydroxyibuprofen from extracts of Ibu-1 supernatant, including retention time (Fig. A1.2). The fragmentation patterns clearly demonstrated these to be acetylated metabolites, indicating that all of the hydroxyl groups were of an aromatic nature. As with the pure culture study, the ring-hydroxylated metabolites were detected only in the 3-fluorocatechol treatment, again suggesting a *meta*-cleavage pathway.

In addition to the ring hydroxylated intermediates, two mass spectra consistent with 2-(4-(2-methylhydroxypropyl)phenyl)propionate (15.28 and 16.31 min.) (hydroxyibuprofen) and one consistent with 2-(4-(2-methylpropionyl)phenyl)propionate (carboxyibuprofen) (16.52 min.) were detected in both treatments (Table A1.1). Similar mass spectra identified as these ibuprofen metabolites have been identified by other researchers (Buser, Poiger et al. 1999; Winkler, Lawrence et al. 2001; Zwiener, Seeger et al. 2002). Two spectra consistent with hydroxyibuprofen were identified in this study, presumably representing isomers.

Table A1.1. Ion abundance data from mass spectra obtained for previously identified ibuprofen metabolites isolated from sewage sludge

Name	retention time (min.)	mass (relative abundance)
Hydroxyibuprofen	15.24	178(71) 119(100) 118(95) 117(73) 91(72) 59(42)
Hydroxyibuprofen	16.31	178(63) 161(25) 119(21) 118(30) 117(32) 91(16) 84(100) 59(2)
Carboxyibuprofen	16.52	264(28) 233(9) 205(100)

Although ibuprofen metabolites with side chain modifications such as these have been previously reported in environmental samples, we also detected unique, ring-hydroxylated ibuprofen metabolites identical to those detected in the supernatant of *Variovorax* Ibu-1 pure cultures. It is possible that these ring-hydroxylated metabolites were not detected previously because they are an artifact of the high concentrations of ibuprofen employed in this study. However, the techniques employed in earlier studies were not capable of detecting such catechols. In this report, 3-fluorocatechol was used to inhibit *meta*-cleavage, resulting in an accumulation of catecholic metabolites, while aqueous acetylation allowed for their detection and characterization via GC/MS. It is

1 important to note that the combination of these two techniques was necessary, as neither
2 technique alone was be able to reveal the presence of the catecholic metabolites.
3 To date, no reports have been published detailing bacterial growth on ibuprofen or
4 suggesting that environmental microbes may metabolize ibuprofen via ring-
5 dioxygenation and subsequent ring-cleavage. While the concentrations of ibuprofen used
6 in this study are far in excess of those detected in the environment, this study
7 demonstrates the biological potential for ibuprofen degradation via direct oxidation of the
8 ring followed by further metabolism using a putative *meta*-cleavage pathway. Such a
9 degradation pathway is one mechanism that may account for a portion of the biological-
10 removal of ibuprofen from wastewater treatment plants (Buser, Poiger et al. 1999) and
11 laboratory reactors (Winkler, Lawrence et al. 2001; Zwiener, Seeger et al. 2002) that has
12 previously gone uncharacterized.
13

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APPENDIX 2

E. COLI EPI00 PFOS3G7 METHODOLOGICAL CONSIDERATIONS

The metabolic performance of *E. coli* epi300 pFOS3G7 proved to be very inconsistent throughout the project. The ibuprofen and phenylacetate metabolic pathway encoded by the *ipf* genes present on the fosmid showed widely different activity rates from week to week and month to month, often forcing a complete shutdown of particular routes of inquiry. Throughout the project, several attempts were made to identify the causes of the erratic behavior. The logic, methods, and results of each line of inquiry are outlined briefly below.

The effect of pH on fosmid activity

Even at it's most optimal, *E. coli* epi300 pFOS3G7 metabolized ipf and paa at remarkably slow rates, often taking four days to achieve the metabolism of a few milimolar of substrate. Mature stationary-state cultures might begin to show drastic deviations from optimal physiological pH; considering that uptake of ibuprofen was shown to be possibly dependent upon passive diffusion followed by coenzyme A ligation (Chapter 4), a rising pH may negatively impact metabolic rate. In order to address this possibility, attempts to stabilize and lower the pH were made.

Methods

Culture media was inoculated from overnight LB cultures into LB with 25ppm chloramphenicol, 2mM arabinose, and 100 ppm ibuprofen with the following modifications.

1. unbuffered (control) (pH 7)
2. 25mM HEPES, pH unmodified (7)
3. 25mM HEPES, reduced to pH 6 with 4ul / 5ml ¼ concentrated sulfuric acid

Ibuprofen concentration was measured at 24 hours via standard HPLC protocols.

Results

At 12 hours, the pH was;

1. pH 8
2. pH 7-7.5
3. pH 7-7.5 (indistinguishable from the non-acidified samples)

18 hours;

1. pH 8.5 (note the increase)
2. pH 7-7.5
3. pH 7-7.5 (slight difference is noticeable)

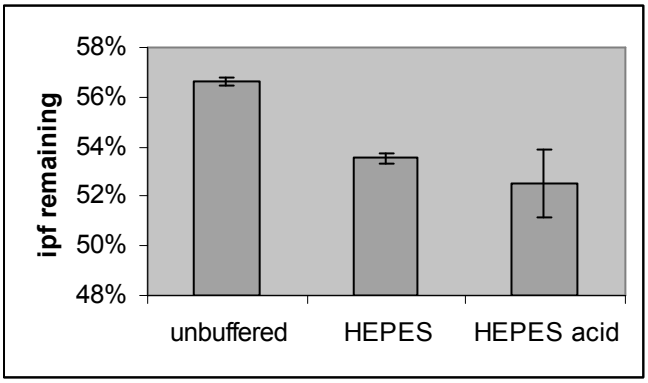


Figure A2.1. Percent ibuprofen remaining after growth of *E. coli* pFOS3G7 in LB media with or without 25mM HEPES buffer and with or without additional acidification of the media to pH6. Y-axis amplified to demonstrate the slight differences.

Conclusions

There is a clear but small effect of pH control on ibuprofen levels. OD600s were measured at 24 hours; no differences were found, though it is possible that a 5% difference (such as the difference in remaining ibuprofen between the cultures) would not have been detected. PIPES, because it buffers in the pH 6.5 range, might be more appropriate. The small size of the effect of buffering on metabolism makes this approach non-ideal. Further acidification of the media and use of a more ideal buffer could be a useful approach for optimizing metabolic rate.

Fosmid aeration

At the onset of the project, a vertical drum shaker was used for aerating fosmid cultures in snap cap falcon tubes. At some point, the drum shaker was no longer in the lab and the cultures were thenceforth incubated in snapcaps at a 45 degree angle with

vigorous shaking. A careful reading of the protocol for the fosmid kit revealed that Epicentre mentions that oxygen levels have a strong effect on fosmid copy number up-regulation by arabinose (p.16, (Epicentre)), though no explanation is given. In order to investigate the impact of aeration on metabolism by fosmid-cloned genes, a more thorough aeration protocol was tested against the traditional protocol.

Methods

Culture media was LB with 25ppm chloramphenicol, 10mM arabinose, and 500 ppm ibuprofen. Two different aeration methods were compared; 15ml snapcap vials versus 50ml culture tubes, both containing 5ml of culture shaken at 45 degree angle at 37 degrees centigrade. For rapid visualization of catechol product, 1.5mM ferric chloride was added to the cultures. Ibuprofen concentration was measured at 24 hours via HPLC.

Results

At 18 hours there was no visible difference in the cultures. At 24 hours, the conical cultures were remarkably dark.

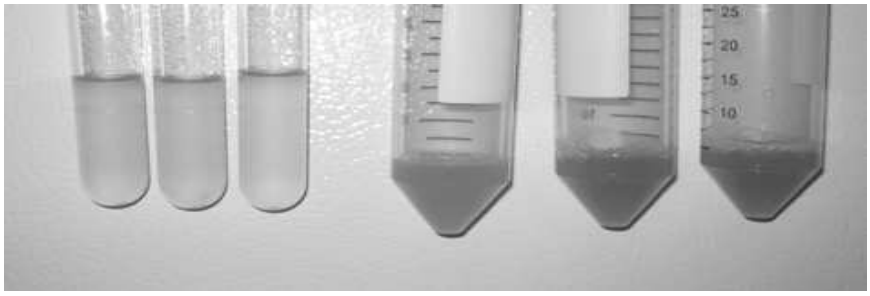


Figure A2.2. Growth of *E. coli* pFOS3G7 in LB media with addition of 10mM arabinose, 1.5mM ferric chloride, and 500ppm ibuprofen in two different types of vessel; falcon tube on the left and 40mL conical on the right.

transferring the snapcap cultures to conicals for visualization purposes clearly showed the difference in product levels (Figure A2.2).

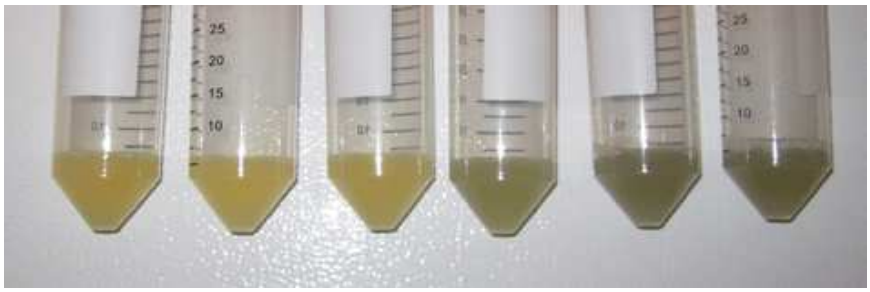


Figure A2.3. Same cultures as shown in Figure A2.2 but with the falcon tube grown culture transferred to conicals for the sake of visual comparison.

Centrifuging a single sample of each for the sake of concentrating the dark iron-catecholic pigment made the visual results more dramatic (Figure A2.4)

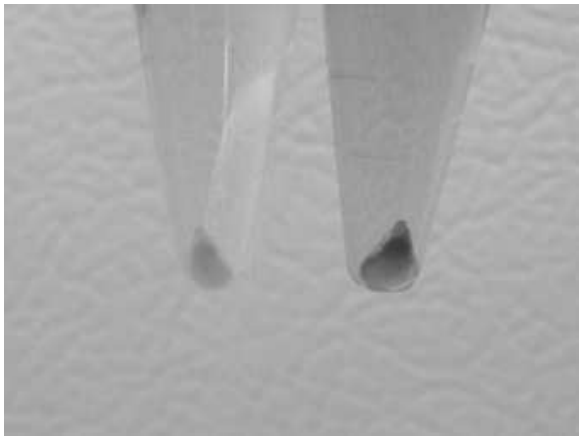


Figure A2.4. One of each culture set shown in figures A2.2 and A2.3 centrifuged in order to concentrate the dark catecholic polymer.

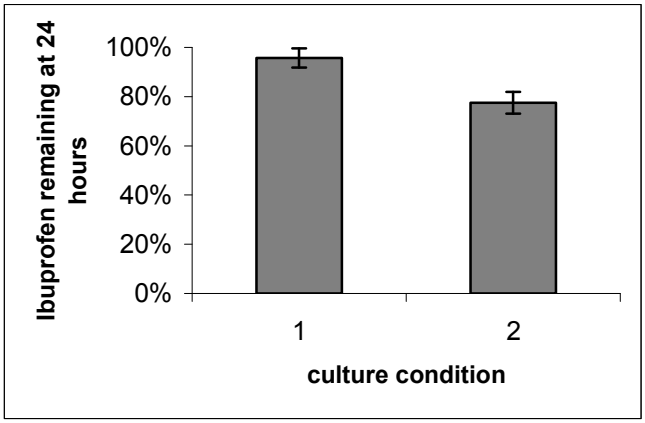


Figure A2.5. Percent ibuprofen remaining in *E. coli* pFOS3G7 cultures grown in falcon tubes (1) or 40mL conicial vials (2).

Conclusions

The snapcap cultures had barely begun to metabolize ibuprofen while the conical cultures had metabolized somewhere in the range of 100ppm of the ibuprofen initially present. Oxygen level clearly had a strong effect on overall metabolic activity, presumably through fosmid copy-number up-regulation, though dependence on oxygen of the dioxygenase step could have also been responsible. It is likely that the higher levels of activity observed during the initial stages of the project were due to much more vigorous aeration of the cultures by the vertical drum shaker.

Addressing possible instability of the fosmid

While the two assays above addressed optimization of the system and did reveal a possible reason that the system performed more robustly during the initial stages of the project when a drum shaker was used, the erratic behavior of the system on a shorter term basis is still unclear. Other researchers using fosmid-based systems have reported similar

erratic behavior (Abbie Wise and Anthony Hay, personal communication) leading to the suspicion that fosmids are particularly prone to mutagenesis or recombination. Additionally, sequencing of pFOS3G7 revealed it to be peppered with transposases (two of which are shown flanking the core *ipf* genes in figure 3.20, the locations of others are outlined in Appendix 5), suggesting that the nature of the insert DNA itself might make it prone to reorganization and possible reduction of activity. Such a mutagenic/recombinative loss-of-function might actually be selected for given the apparently toxic nature of certain elements of the *ipf* pathway (See Appendix 4). In order to address this hypothesis, three individual *E. coli* epi300 pFOS3G7 clones were cultured separately and their activity levels compared to one another.

Methods

E. coli epi300 pFOS3G7 was streaked from the culture collection. Three individual colonies were inoculated into LB, grown overnight, and 100 ppm ibuprofen with 2mM arabinose. Ibuprofen remaining at four days was measured via HPLC.

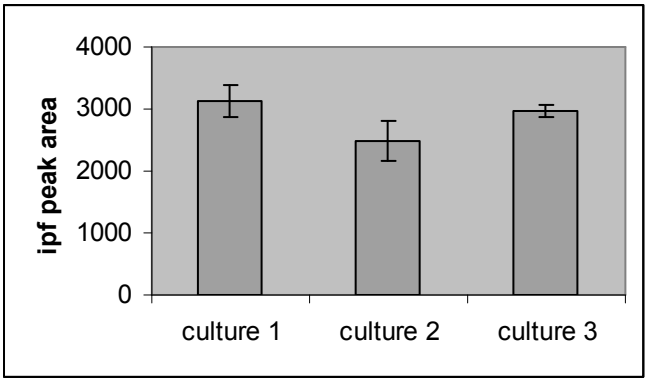


Figure A2.6. Comparison of remaining ibuprofen in *E. coli* pFOS3G7 cultures descended from different colonies.

Results

Culture 2 degraded significantly more ibuprofen than the other two as determined by unpaired students t-test (Figure A2.6).

Conclusions

The results demonstrate the erratic behavior of the pFOS3G7 system. In this case, it appears that two of three cultures develop lower activity levels than would be expected in accordance with the hypothesis.

Complete system shutdown

In November of 2010, *E. coli* epi300 pFOS3G7 and all derivatives nearly completely ceased to function in any capacity. Virtually every aspect of the system was varied in hopes of identifying the culprit for the across-the-board loss-of-function including;

- Ultra-pure mili-Q water versus distilled water
- Stocks of every media component including sodium chloride, tryptone, arabinose, chloramphenicol, yeast extract
- Culture vessel material; glass versus plastic
- Culture temperature was tested and verified to be 37 degrees centigrade
- Assuring that cultures were as freshly out of freezer stocks as possible

Despite aggressive testing, the cause of the shut-down was not identified, bringing the project to a temporary halt.

REFERENCES

Epicentre CopyControl™ Fosmid Library Production Kit with pCC1FOS™ Vector.

APPENDIX 3

IDENTITY AND INITIAL CHARACTERIZATIONS OF OTHER GENES OF INTEREST IN *SPHINGOMONAS* IBU-2 FOSMID LIBRARY CLONES

Abstract

Additional sequencing of pFOS3G7 and pFOS4F6, a clone that is capable of ibuprofen metabolism but does not accumulate catechols, revealed several genes whose sequence identities suggested possible involvement in the *ipf* pathway in some capacity. Herein, *in silico* analyses of the the putative gene products are presented along with initial inquiries into what role they may play in ibuprofen metabolism. The gene names should be regarded as tentative since in no case was the role of the gene or its requirement for metabolism or transport substantiated. Briefly, elements of an aromatic dioxygenase complex, both the reductase (IpfH) and the ferredoxin (IpfI) were identified. Knockout of *ipfH* slowed substrate disappearance. The two genes were cloned into *E. coli* epi300 pJ25(*ipfABDEF*) but failed to complete the pathway. An aromatic 1,2-dioldehydrogense gene *ipfL* was knocked out with no consequence on pFOS3G7. A putative regulatory element *ipfR* was identified and initial HPLC data suggests that it plays an inhibitory role. Two other elements of interest, an enoyl-CoA hydratase (*ipfY*) and an acyl-CoA racemase (*ipfT*) were identified via sequence analysis, though initial data suggests that they do not play an essential role in the *ipf* pathway.

Interestingly, *ipfT* shares remarkable sequence similarity with human alpha-methylacyl-CoA racemase, an enzyme that performs a fortuitous chiral inversion of ibuprofen.

Ferredoxin Reductase ipfH

Please see Chapter 3 for a description of this ORF.

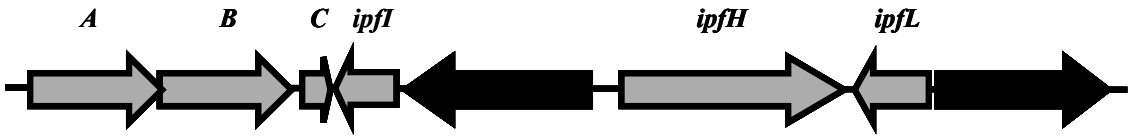


Figure A3.1. ORFs found on the SeqMan assembly contig containing ferredoxin reductase *ipfH,* ferrdoxin *ipfI,* and diol-dehydrodgenase *ipfL.* Black arrows represent sequences with high similarity to conserved transposases. Regions A, B, and C have similarities to 4-hydroxy-2-oxovalerate aldolase, 4-oxalocrotonate decarboxylase, and 4-oxalocrotonate isomerase.

Plant-like ferredoxin ipfI

Near *ipfH,* a small ORF was located via random sequencing (Figure A3.1). No transposon mutant with an insertion in this small 333bp ORF was found. Conserved domain analysis of the translated ORF located a 2Fe-2S ferredoxin conserved domain; alignment with stereotypical members of the group revealed that each of the conserved iron-coordinating cysteines is present (Figure A3.2).

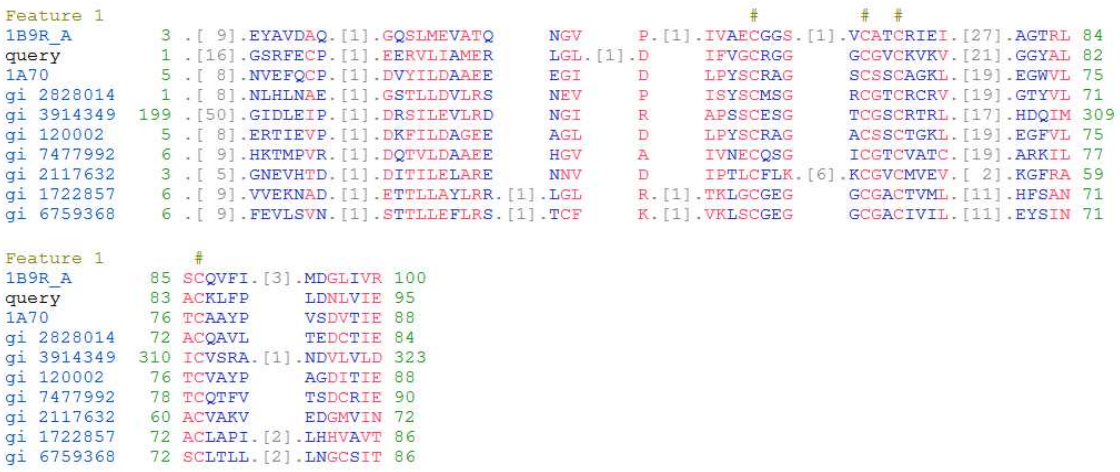


Figure A3.2. Alignment between ferredoxin reductase IpfH ("query") and several other ferredoxin reductases found in the NCBI database showing the four conserved iron-coordinating cysteine residues indicated by "#".

Addition of the electron transport genes to sublconed ipfABDEF *(pJ25)*

****Important Note** The remainder of this section on *ipfHI* was created before the modification of the methodology that allowed for strong conversion of substrates to corresponding catechols by pJ25 pGEM:*ipfHI*.**

Consideration of the *ipfH* knockout data, the proximity of *ipfI*, and the sequence characteristics of both led to the hypothesis that the two gene products are the electron transport chain of the dioxygenase IpfAB. The lack of the presence of these two genes on pJ25 might explain the very poor catechol generation displayed by pJ25-expressing clones. To address this hypothesis, *ipfH* and *ipfI* were targeted for cloning. In order to place both genes on the same plasmid despite their distance from one another (it was not known at the time that they were so closely located to one another), a PCR strand overlap

extension (SOE) strategy was employed. SOE employs PCR primers with additional 5' homology overlaps between the two PCR products that allows for artifical joining of the two PCR products. An initial round of PCR using a 50C anneal and 1.5 minute extension with a pFOS3G7 template and the SOE primers (Table A3.1) was successful (Figure A3.3)

Table A3.1. Primers designed for use in the creation of novel Ibu-2 fosmid library constructs. Italicized nucleotides indicate the manufactured overlap sequence for use in the strand overlap extension combination of *ipfI* and *ipfH*, while the lower-case nucleotides indicate sequence that is specific for the pKD4-borne kanamycin resistance insertion cassette used in the creation of gene-replacement mutants via the lambda red system.

primer name	primer sequence
ipfFeDox F sew	TAC CGC CGA GCA GGA ATA TTA CAG CCG CGA CA
ipfFeDox R sew 2	GAA GCG GGT ACA TTC CTC CAG ACG GTC CTC
ipfFeDoxRed F sew	*GGA GGA ATG TAC CCG CTT C*AC GCA CAC AAT CTA
ipfFeDoxRed R sew	TTT CAC CGC AGG CCT ATG CCG C
ipfLlambdaF	ACGGCGCGCCTGAGAGGCCGAGATCCAGCGCTCCTT gtgtaggctggagctgcttc
ipfLlambdaR	ACGGAAGAGGGTGGGCGCCTTGGACGCCCCCTCAGA atgggaattagccatggtcc

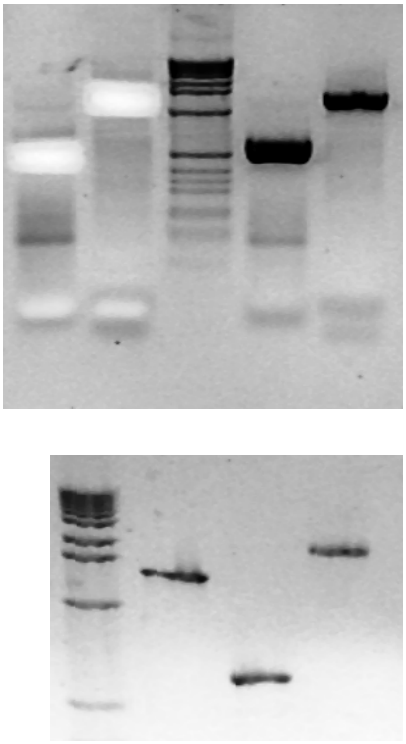


Figure A3.3. The FedoxSOE project. The sizes of the *ipfI* and *ipfH* amplicons are shown above, while the successful strand overlap extension product is shown on the lower right.

The products, which were of the predicted size, were gel purified and used as template for three cycles of primer-less PCR under the same conditions. IpfFeDox F sew and ipfFeDoxRed R sew were then added and PCR performed under the same conditions, yielding a combined product of the predicted size (Figure A3.3). The SOE product was t-cloned into pGEMt-easy, sequence verified, and transformed into *E. coli* epi300 pJ25. The sequence was searched *in silico* for the presence of any *E. coli* –recognizable transcriptional terminators, of which none were present.

While *E. coli* epi300 pJ25 pGEM:*ipfHI* did turn a slightly darker color when induced with IPTG (Figure A3.4), no detectable catechols were generated as determined by ferric chloride assay and HPLC analysis (method described in Chapter 2); the dark color was likely due to high expression of the ferredoxin or to interaction of the electron transport proteins with the ferric chloride present in the diagnostic media.

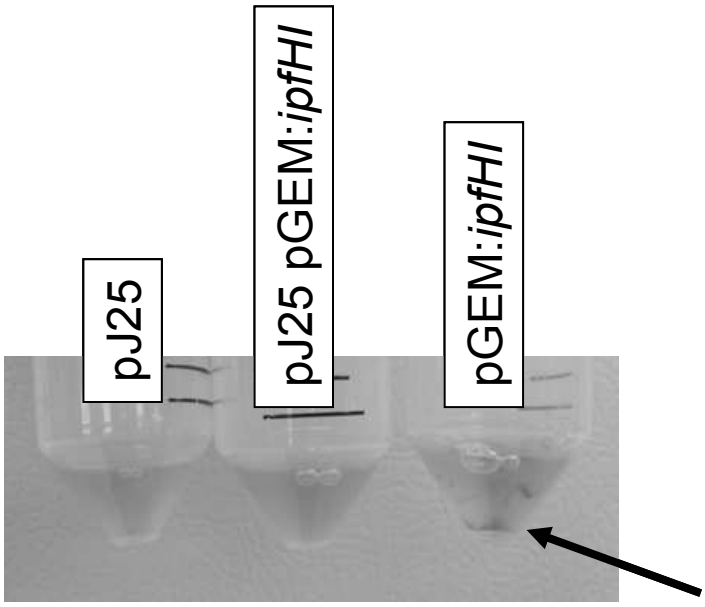


Figure A3.4. *E. coli* epi300 pJ25, *E. coli* epi300 pJ25 pGEM:*ipfHI*, and *E. coli* JM109 pGEM:*ipfHI*, all with IPTG induction, grown in LB with 500 ppm ibuprofen and 1.5mM ferric chloride. The slightly dark color that appeared in the pJ25 pGEM:*HI* culture (poorly visible in this photograph) did not pellet in the same manner as the catecholic polymers produced by *E. coli* epi300 pFOS3G7 (Figure 3.18). pGEM:*ipfHI* expression in JM109 led to a dark coloration in the culture media and in deceased cell material (indicated by arrow).

HPLC analysis for accumulation of 2-hydroxyphenylacetic acid, presumably a byproduct of the abiotic degradation of the 1,2-diol product of dioxygenase activity, showed that *E. coli* pJ25 pGEM:*ipfHI* accumulated the 2-hydroxy to a higher extent when cultured with 1mM IPTG (Figure A3.5) and that the phenylacetate substrate concurrently disappeared at a faster rate. However, given that the pBBR1mcs plasmid that is the backbone of pJ25 and the pGEMt-easy plasmid that is the backbone of the pGEM:*ipfHI* plasmid both have a *lacZ* promoter, it is not clear which aspect of the pathway the IPTG was inducing. However, this data strongly suggests that the dioxygenase system is functioning in *E. coli* pJ25 pGEM:*ipfHI*, especially given that induction of the cloned elements speeds the production of this hydroxylated byproduct. However, this data was

poorly reproducible; in subsequent assays the pJ25 pGEM:*ipfHI* construct behaved differently until the methodological adjustments described in Chapter 3, namely using a higher concentration of chloramphenicol and delaying addition of substrate until 24 hours of growth.

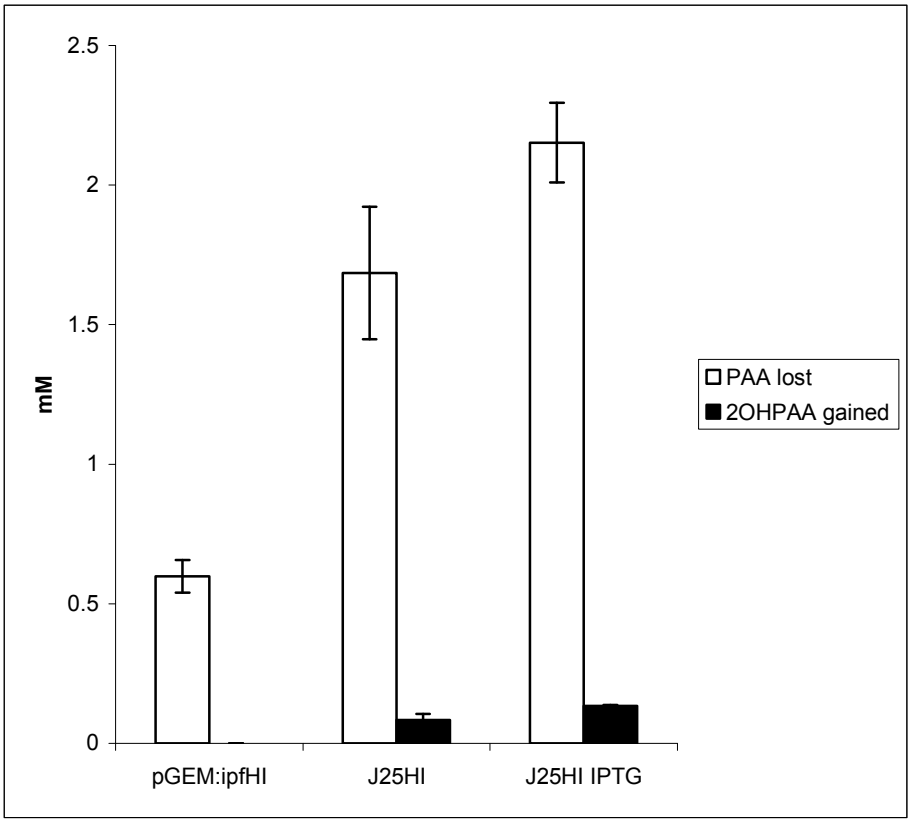


Figure A3.5. 2-hydroxyphenylacetete accumulation and phenylacetate disappearance by two *E. coli* JM109 pGEM:*ipfHI*, *E. coli* epi300 pJ25, and *E. coli* epi300 pJ25 pGEM:*ipfHI*. Effect of *lacZ* induction on the metabolic behavior of J25F was examined by addition of 1mM IPTG. All data points are significantly different from one another as determined by t-test ($p<0.05$)

In later assays performed a year later using the same strains and techniques described above but with the addition of a vectorless *E. coli* epi300 negative control yielded different results. *E. coli* epi300 pJ25 pGEM:*ipfHI* cultures degraded 1.4mM of

phenylacetate and accumulated 0.7mM (+/- 0.05mM) catechol as detected by HPLC analysis of two day old cultures (Figure A3.6) and dark color generation by two weeks (Figure A3.7) while the other strains did not.

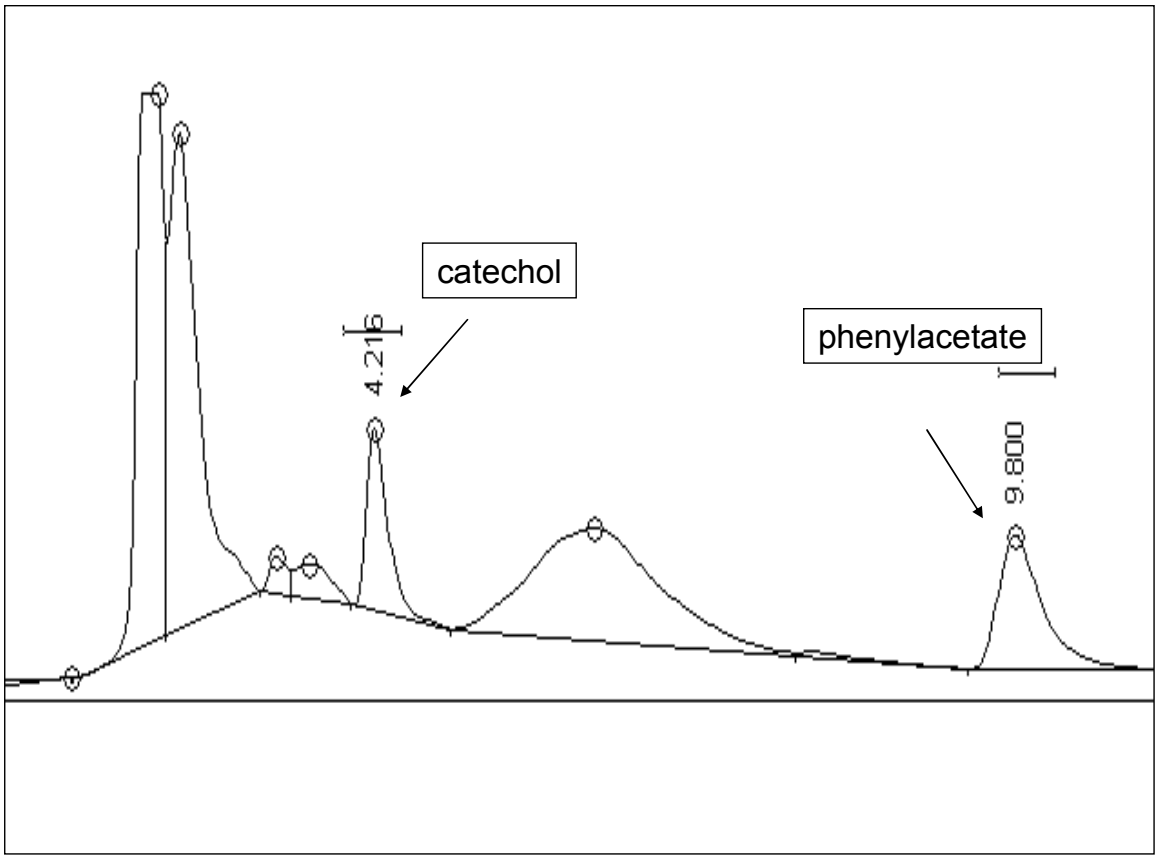


Figure A3.6. HPLC of *E. coli* epi300 pJ25 pGEM:*ipfHI* grown in LB with 5mM phenylacetate for two days. Strains with single or no vector accumulated no detectable catechol. This culture went on to turn dark brown as seen in Figure A3.7.

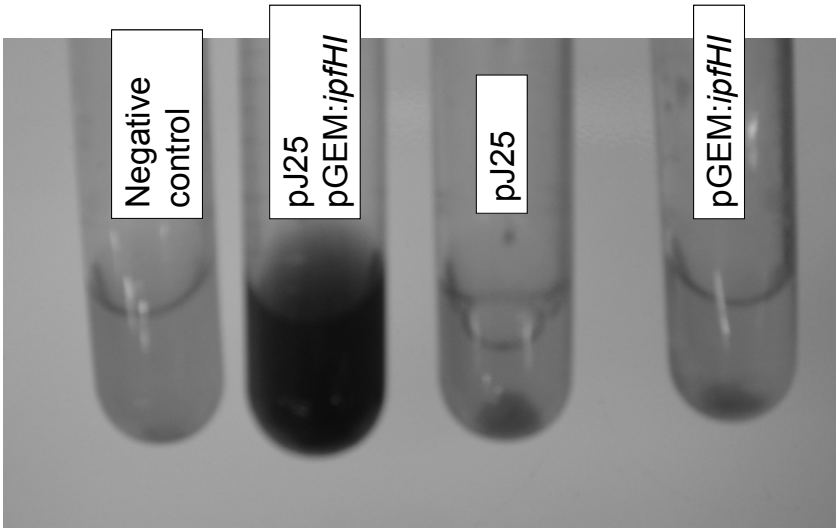


Figure A3.7. *E. coli* pJ25 pGEM:*ipfHI* after two weeks incubation in LB media with 5mM phenylacetate compared to *E. coli* with only one or neither of the plasmids.

Ultimately, this strong catechol generation was reproduced by using four times as much chloramphenicol and by spiking stationary phase cultures with substrate instead of growing cultures in the presence of substrate from inoculation. In addition to the phenylacetate and ibuprofen metabolism presented in Chapter 3, *E. coli* epi300 also metabolized *m*-tolylacetate and 2-phenylacetate to 3-methylcatechol and catechol respectively. For direct visualization of catechols, ferric choride was added to 150ul of culture to a final concentration of 1.5mM in 96-well plate format. HPLC was used to directly quantify substrate and catechols. 40:60 methanol:40mM acetic acid running buffer was used for separation of phenylacetate (10.6 minutes), catechol (4.3 minutes), and 3-methylcatechol (7.8 minutes). 50:50 methanol:40mM acetic acid was used to separate *m*-tolylacetate (11.8 minutes) and 2-phenylpropionate (11 minutes). A detection wavelength of 220nm was used for the aromatic acids while a detection wavelength of 280nm was used for the catechols.

The activity of *E. coli* epi300 harboring both pJ25 and pGEM:*ipfHI* towards *m*-tolylacetate and 2-phenylpropionate was consistent with the results presented in Chapter 2 (Figure A3.8). The dual vector system degraded approximately 0.55 mM of the *m*-tolylacetate added and accumulated 0.18 mM of 3-methylcatechol (Figure A3.9). The dual vector system also degraded 0.62 mM 2-phenylpropionate and accumulate 0.19 mM catechol (Figure A3.10).

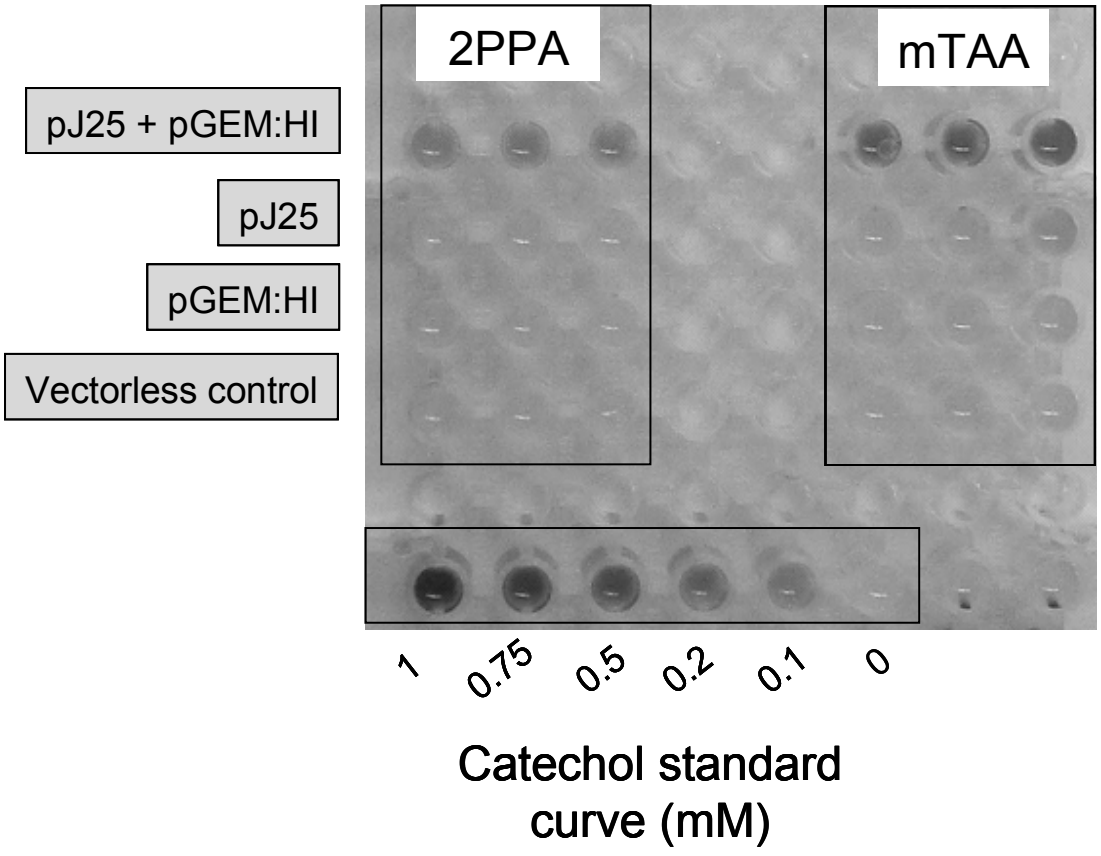


Figure A3.8. 150ul of *E. coli* epi300 harboring the indicated vector exposed to either 2-phenylpropionate (2PPA) or *m*-tolylacetate (mTAA). 1.5 mM ferric chloride has been added as a catechol indicator. A standard curve of catechol with 1.5 mM ferric chloride is presented for reference.

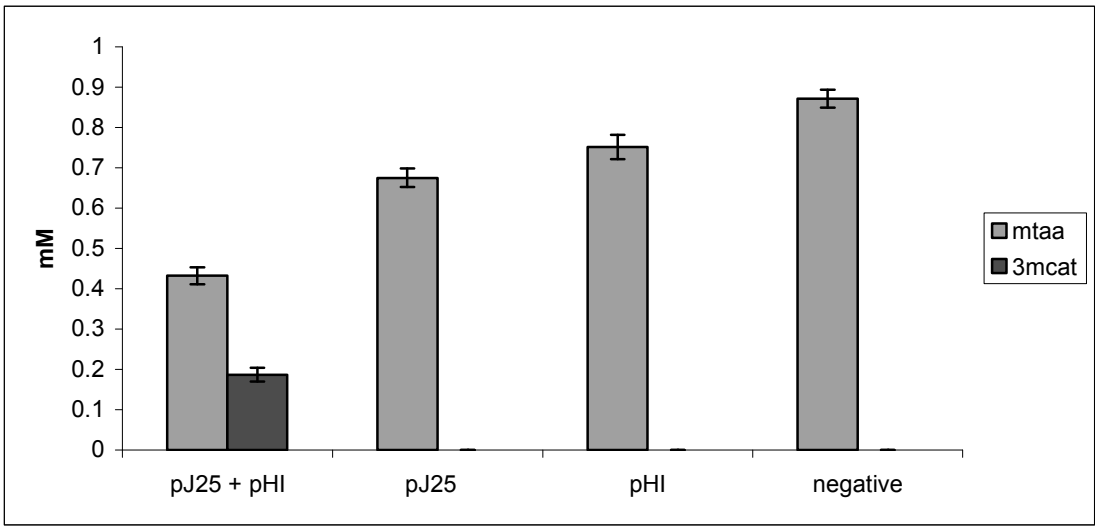


Figure A3.9. *m*-tolylacetate and 3-methylcatechol concentration in *E coli* epi300 cultures harboring pJ25 and/or pGEM:*ipfHI* (pHI) following 18 hours of incubation 1 mM ibuprofen.

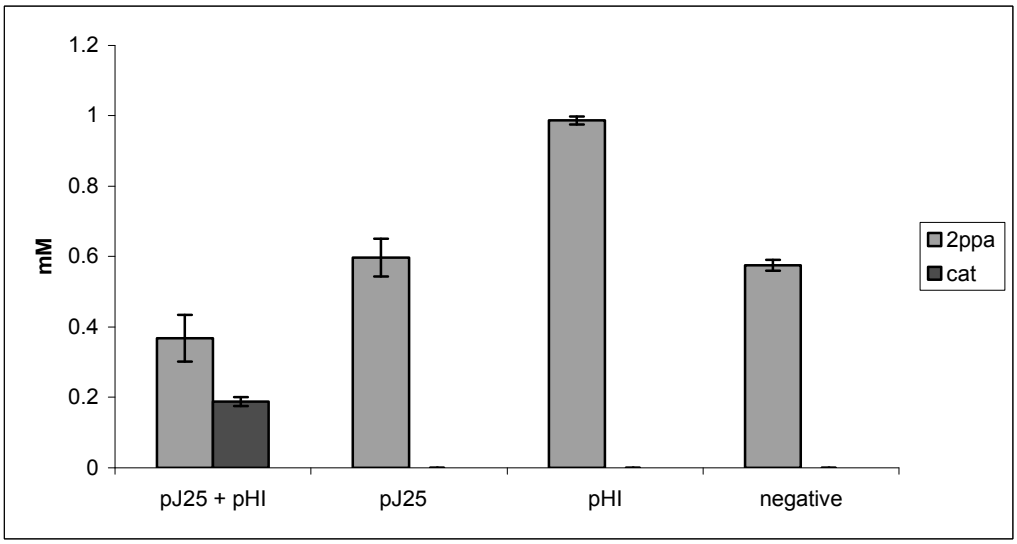


Figure A3.10. 2-phenypropionate and catechol concentration in *E coli* epi300 cultures harboring pJ25 and/or pGEM:*ipfHI* (pHI) following 18 hours of incubation 1 mM ibuprofen.

Additional Genes of Interest

The early unreliability of the *E. coli* pJ25 pGEM:*ipfHI* system suggested that a further gene product might be required for efficient function of the *ipf* pathway. In order to address this now defunct hypothesis, available pFOS3G7 and pFOS4F6 sequence information was scoured for possible genes of interest, of which four, detailed below, were identified. The characteristics of these putative genes and attempts to create and/or characterize knockouts of each are detailed below.

***Diol-dehydrogenase* ipfL**

Aromatic dioxygenase pathways often employ a diol-dehydrogenase step following the initial dioxygenation. While the putative *ipf* pathway does not allow for the involvement of a classical diol-pdehydrogenase, the possibility remains that a modified protein serves a similar function in Ibu-2. Indeed, adjacent to *ipfH,* an ORF was identified with high similiarity to x*ylL,* a diol-dehydrogenase that acts on the 1,2-diol of benzoate (Figure A3.11). The existing data regarding the activity of *E. coli* pJ25 pGEM:*ipfHI* suggested that there might be an additional required protein at the later stages of the pathway.

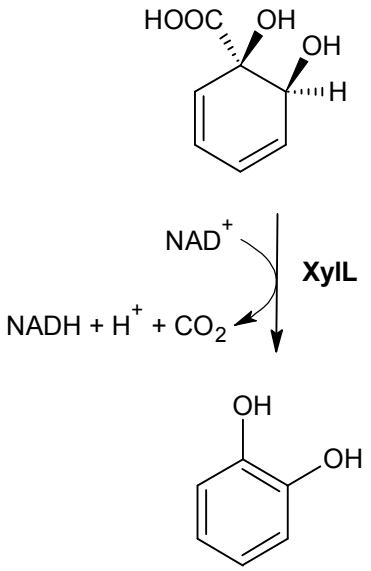


Figure A3.11. General reaction performed by the diol-dehydrogenase XylL.

As no existing sequenced transposon mutants had insertions in this small 432bp ORF, the lambda red system (Chapter 3) was used to create an *ipfL* knockout in pFOS3G7. pKD4 *ipfL* primers and a primer flaking the *ipfL* ORF (for verifying successful insertion) were designed (Table 3.7) and incorporation into pFOS3G7 was successful (Figure A3.6), the ipfLlambdaF and ipfLR primer set yielding a 2.2 kb product indicative of successful incorporation of the pKD4 kanamycin cassette. Screening with two flanking *ipfL* gene primers was consistent with gene replacement. However, screening the insertion clones on diagnostic plates (LB with 100 ppm p-toluidine, 100ppm ibuprofen, and 1.5mM ferric chloride, Figure A3.12) clearly showed that the pathway was still intact. Liquid culture assays with proper control strains clearly confirmed this result (data not shown).

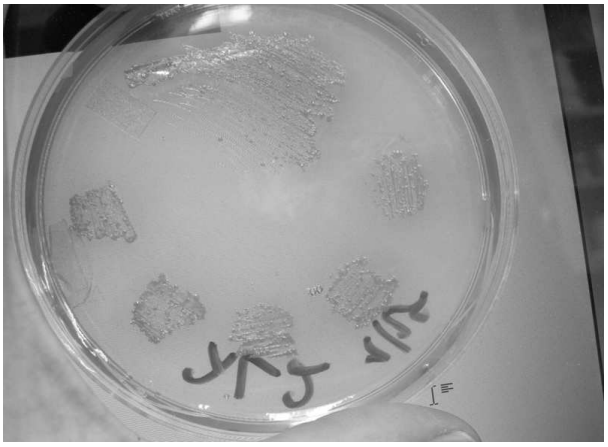


Figure A3.12. Dark catecholic accumulation by *E. coli* epi300 pFOS3G7 with *ipfL* replaced by the pKD4 insertion cassette (confirmed by PCR in Figure A3.13). The media consists of LB with 100 ppm ibuprofen and 1.5mM ferric chloride.

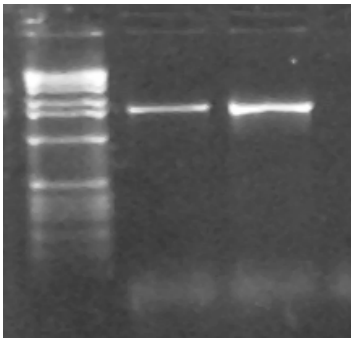


Figure A3.13. PCR screening of several *E. coli* epi300 pFOS3G7 *ipfL* pKD4 insertion cassette mutants using primers that flank the *ipfL* gene showing successful gene replacement; *ipfL* is 432 bp while the insertion cassette is approximately 1.6kb.

The presence of a *xylL* analog directly adjacent to a gene strongly suspected to be part of the *ipf* pathway in addition to the data leading to the conclusion that an additional late-upper-pathway gene is required for efficient metabolism proved too good to be true. Knocking out *ipfL* did not lead to loss of function to any degree. However, the possibility remains that there is another copy of *ipfL* present on pFOS3G7 in a different genetic context that the flanking primers might not be able to detect nor the pKD4 *ipfL* primers able to knock out (given that they target flanking DNA for recombination). The still

leaves open the possibility that such a gene is required for metabolism. This hypothesis can be tested by cloning *ipfL* into *E. coli* pJ25 pGEM:*ipfHI* in order to see if the pathway is completed; given that in *E. coli* pJ25 pGEM:*ipfHI*, both pBBR1 and pGEM plasmid systems are present, the pOFX low copy number expression system may prove useful.

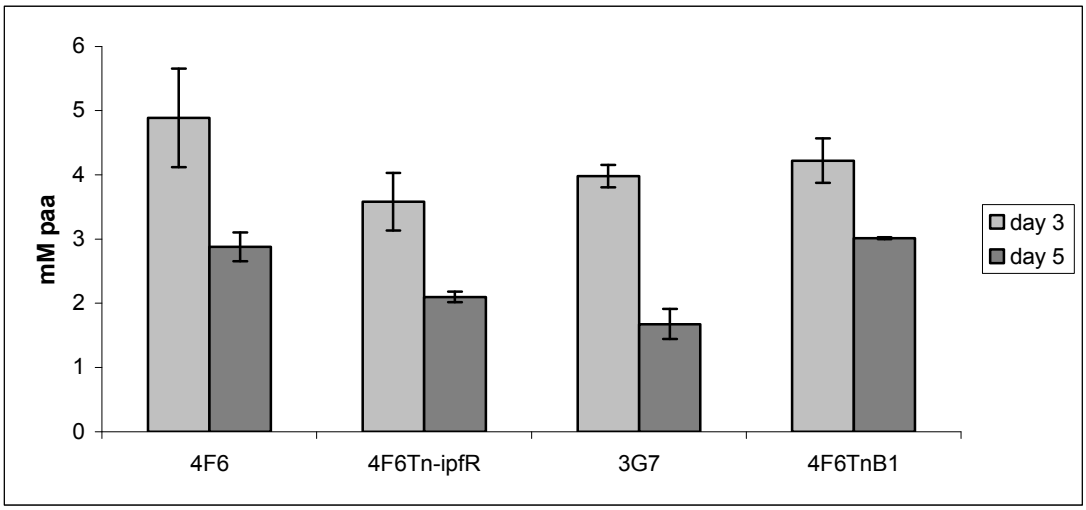
***AAA+ ATPase* ipfR**

Approximatly 3.5kb downstream of *ipfF* a AAA+ ATPase domain was located. The sequence data in this region was of fairly low quality, prohibiting identification of the full ORF. The identified ORF at present is represented by approximately 1kb. The identity with the conserved AAA+ ATPase domain via BLAST conserved domain search was quite confident (E-value = $3x10^{-31}$). The AAA+ ATPase domain is a molecular motor that couples ATP hydrolysis to mechanical motion. This domain is found in all domains of life in a very wide variety of proteins, often those with proteolytic or DNA regulatory roles (Hanson and Whiteheart 2005). While the *ipfR* full ORF was not identified, the ready availability of a transposon mutant with an insertion in *ipfR* coupled with its possible regulatory role made an initial screening of mutant activity fairly simple.

pFOS4F6 is one of the Ibu-2 pCC1 chromosomal library clones capable of metabolizing ibuprofen and phenyylacetic acid all the way to the corresponding meta-cleavage products. A transposon mutant A3 had already been created by random transposon mutagenesis. *E. coli* epi300 pFOS4F6TnA3 was subjected to metabolic analysis alongside *E. coli* epi300 pFOS4F6, *E. coli* epi300 pFOS3G7, and negative control epi300. Because *E. coli* epi300 pFOS4F6 does not accumulate catechols, visual

ferric chloride assays were not useful. Phenylacetate disappearance and 2-hydroxyphenylacetate were monitored via HPLC and quantified against standard curves (Figure A3.14).

Surprisingly, the HPLC analysis showed that *E. coli* epi300 pFOS4F6Tn:*ipfR* actually metabolized *more* phenylacetate than intact *E. coli* epi300 pFOS4F6. Whereas *E. coli* pFOS4F6 has been known for years to be less active towards phenylacetic substrates than *E. coli* epi300 pFOS3G7, *E. coli* epi300 pFOS4F6Tn:*ipfR* actually reached substrate disappearance rates similar to that shown by *E. coli* epi300 pFOS3G7. It is however important to note that this only represents the first steps of the pathway and that pathway products were not quantified to any degree.



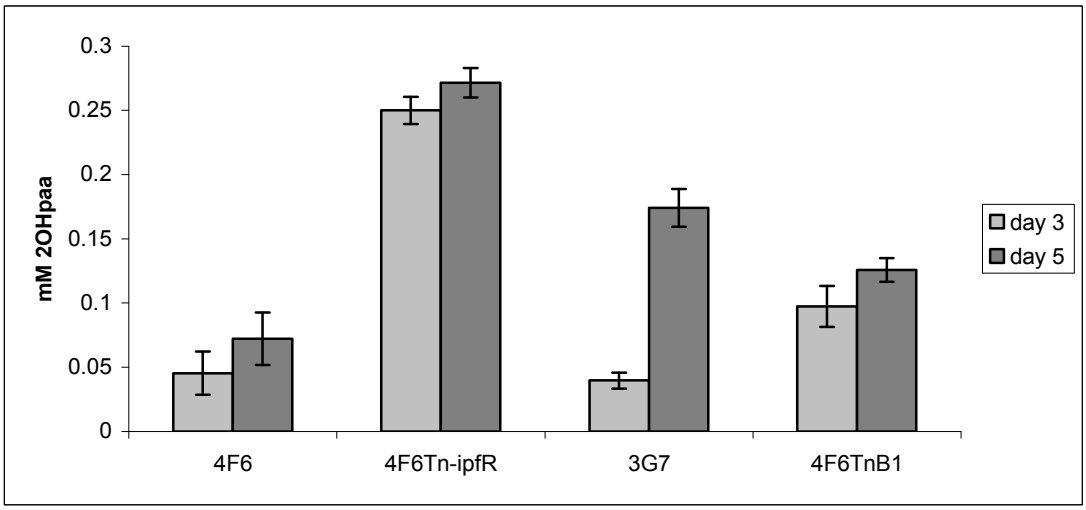


Figure A3.14. the *ipfR* transposon mutant HPLC assay showing increased phenylacetate disappearance (top) and increased appearance of 2-hydroxyphenylacetate (bottom). Also included in the assay was the *ipfY* pFOS4F6 transposon insertion mutant pFOS4F6B1.

In order to make a more detailed statement, resting cell assay with 3-flouorcatecholic meta-cleavage inhibition with measurement of catechol accumulation as described in chapter 2 would be necessary. Strangely, while phenylacetate disappearance in *E. coli* epi300 pFOS4F6Tn:*ipfR* was similar to that shown by *E. coli* epi300 pFOS3G7, accumulation of 2-hydroxyphenylacetate was remarkably higher in the *ipfR* mutant. This may indicate non-ideal expressional control, the result of which might be that the first

few steps of the pathway have been released from regulatory control by disruption of the putative regulatory element *ipfR* resulting in over-production of the diol intermediate at such a rate that more is lost to abiotic dehydration to yield the 2-hydroxy product (Figure A3.14). These results led to the general hypothesis that *ipfR* serves a regulatory function. Two further mutually exclusive hypotheses derive from this initial obvious one.

Hypothesis 1

Hypothesis 1 is that the higher rate of activity in *E. coli* epi300 pFOS4F6Tn:*ipfR* might indicate that *E. coli* epi300 pFOS3G7 does not have an intact copy of *ipfR* which might explain the difference in metabolic rates between the two intact fosmid clones. Note that this hypothesis does not take into account the differences in 2-hydroxy byproduct. However, the hypothesis was fairly simple to address by examining existing sequence information and by testing primers that target the existing *ipfR* ATPase domain gene fragment. Sequence information showed that one end of the pFOS3G7 fosmid backbone appears to end approximately 1000bp downstream of the ATPase *ipfR* domain making the question as to the presence of an intact gene unresolved (Figure A3.15). AAA+ ATPase domain-containing proteins are of highly variable and totally unpredictable sizes given that the C-terminal of these proteins are extremely divergent and substrate/role-specific.

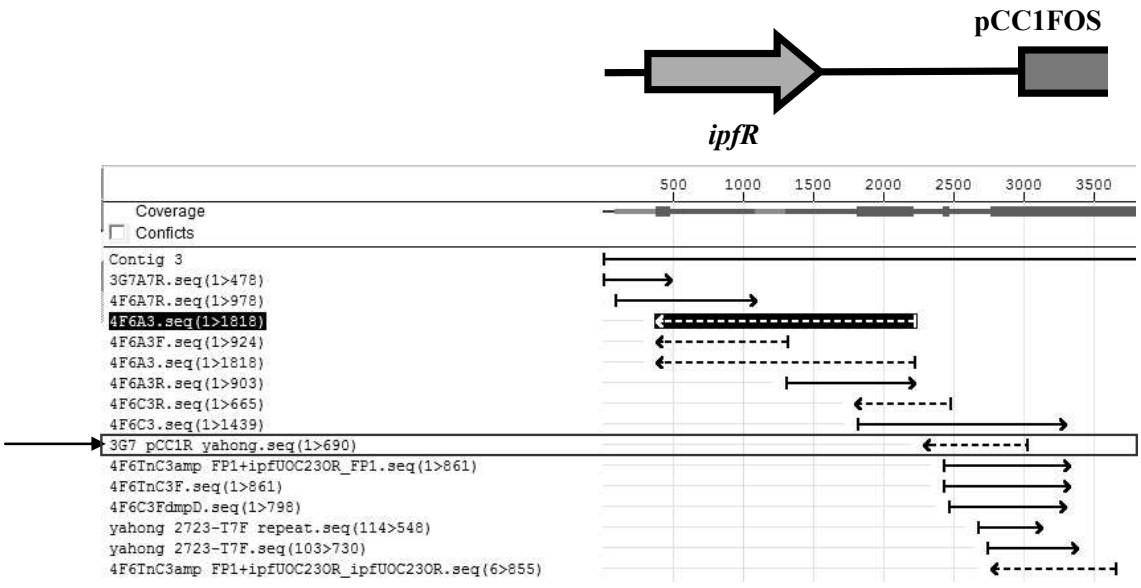


Figure A3.15. SeqMan (DNAStar inc., Madison, WI) screencap of the fosmid library sequence assembly project showing the proximity of the end of the pFOS3G7 DNA insert to the vector; sequence "3G7 pCC1R yahong.seq" begins at the end of the vector.

To further address the question, primers flanking the domain were created (Table A3.2) and used on *E. coli* epi300 pFOS3G7 and *E. coli* epi300 pFOS4F6 (Figure A3.15). In accordance with the sequence data, *E. coli* epi300 pFOS3G7 responded to the ipfR domain flanking primers. In order to determine whether pFOS3G7 has an intact *ipfR* ORF, more sequence data would be required.

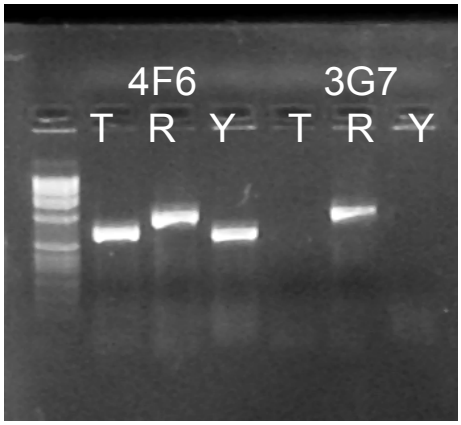


Figure A3.16. PCR of *ipfT*, *ipfR*, and *ipfY* from fosmid pFOS4F6 or pFOS3G7 template.

Hypothesis 2

The second and exclusive hypothesis is that pFOS3G7 encodes an intact IpfR regulatory protein that if knocked out, would lead to more rapid substrate utilization and more rapid byproduct accumulation. To address this hypothesis, the *ipfR* ATPase domain was targeted for deletion using the lambda red system (primers in Table A3.2). Incorporation of the pKD4 cassette into pFOS3G7 was successful, but PCR results continued to suggest the presence of both replaced gene and intact gene despite several round of extra-copy fosmid curing (Figure A3.17). The presence of intact *ipfR* ATPase domain suggests that there is an extra copy of *ipfR* on pFOS3G7.

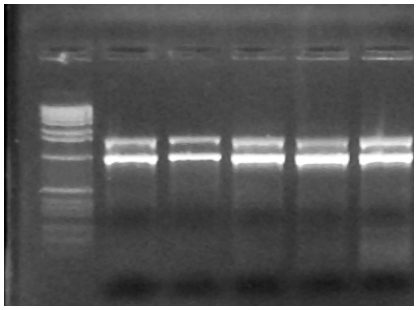


Figure A3.17. PCR of *E. coli* epi300 pFOS3G7 with *ipfR* replaced by the pKD4 kanamycin resistance cassette using primers that flank the region of *ipfR* targeted for deletion. Note that the double bands correspond to both the pKD4 insertion cassette (large band) and intact *ipfR* (small band).

Conclusions

Initial data suggests that *ipfR* AAA+ ATPase domain is part of an inhibitory regulation apparatus. In order to address this properly, more sequence information is required so as to more confidently identify the ORF. Next, the gene could be over expressed in one of the fosmid clones in order to examine whether it has an inhibitory effect on metabolic activity.

Acyl-CoA racemase ipfT *and enoyl-CoA hydratase* ipfY

Two more genes with conserved domain identity that suggests an involvement in aromatic metabolism were located in the pFOS4F6 transposon mutant library. A single *E. coli* pFOS4F6 transposon mutant was identified that had the tranposon insertion in a region dubbed *ipfY* with high BlastX similarity to enoyl-CoA hydratases (Figure A3.18). Upstream of this region was a second region with an acyl-transferase/racemase domain dubbed *ipfT*. The sequence quality in this region was very poor, prohibiting confident

determination of the ORFs. *ipfY* appears to be very near the end of insert DNA on pFOS4F6 (Figure A3.18).

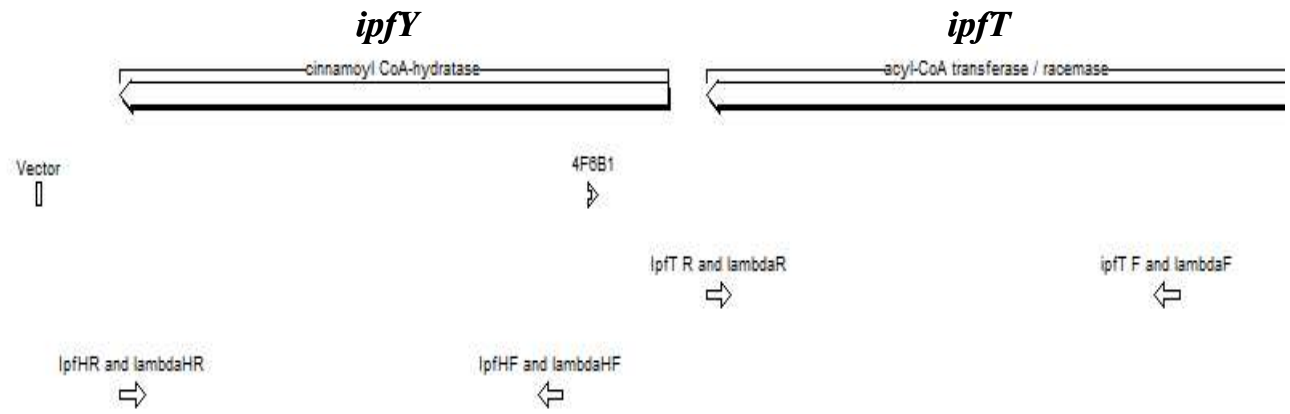


Figure A3.18. Map of partial *ipfY* and *ipfT* ORFs showing the location of the pFOS4F6 vector, the location of the pFOS4F6Tn:*ipfY* transposon isertion, and the targets of the ORF-specific and lambda red pKD4 primers.

ipfY

The gene with highest blast-X similarity (30% identity and 86% coverage) to IpfY was 3-hydroxypropionyl-CoA dehydratase (Teufel, Kung et al. 2009), while wide general similarity was found to many general types of enoyl-CoA hydratases and dehydratases. This general gene family is involved in several types of pathways including general fatty acid oxidation and anaerobic aromatic metabolic pathways for cinnamoyl-CoA and chlorobenzoates (Hamed, Batchelar et al. 2008). An initial screening of the pFOS4F6 *ipfY* transposon mutant suggested that general phenylacetate metabolism was unaffected by inactivation of this gene (Figure A3.14). Screening of pFOS3G7 with primers specific

for an internal region of the gene revealed that it is not present on pFOS3G7 (Figure A3.16), fairly strongly arguing against an essential role in the upper *ipf* pathway. Indeed, the wide applicability of the enoyl-CoA reaction could reflect the involvement of IpfY farther downstream in the lower pathway of the reaction.

ipfT

Upstream of *ipfY,* the sequence contains a putative ORF with a conserved acyl-CoA transferase/racemase domain. CoA transferases generally catalyze the exchange of a CoA group from one acyl-CoA to another carboxylic acid group, a reaction that is not suspected to be relevant to the *ipf* pathway. However, the second general function of this conserved domain in the racemation of chiral acyl-CoAs. Most intriguing is its high similarity to alpha-branched acyl-CoA isomerases found in animals with which it shares 50-65% identity (see human 2-methylacyl-CoA racemase Q9UHK6.2). The methyl-acyl-CoA racemases are responsible for isomerization of bile acids and pristanoyl-CoA in particular (figure 3.8)(Setchell, Heubi et al. 2003) which is particularly interesting given the related putative functions of IpfD and IpfE in the beta oxidatition of alpha-methyl-acyl-CoA compounds. *Perhaps most intriguingly, in humans this protein is responsible for the conversion of (R)-ibuprofen-CoA to (S)-ibuprofen-CoA* (Reichel, Brugger et al. 1997). *Sphingomonas* Ibu-2 growing on ibuprofen as sole carbon and energy source depletes the R enantiomer more quickly than the S (figure 2.1), suggesting that a similar chiral inversion system may be at work. Indeed, in the metabolism of bile acids and pristanoyl-CoA, the beta-oxidative steps are highly enantiomerically-specific, suggesting

that such an inversion of ibuprofen-CoA would be necessary when taking into consideration the analogies between the eukaryotic and Ibu-2 pathways.

There was no characterized pFOS3G7 or pFOS4F6 *ipfT* transposon mutant available for screening. PCR screening of pFOS3G7 with internal primers was negative (Figure A3.16), suggesting that pFOS3G7 does not have a copy of *ipfT* (Figure A3.16). The fact that *Sphingomonas* Ibu-2 is able to metabolize both isomers of ibuprofen (Chapter 2) suggests that there is an isomerase involved in the pathway. The complete metabolism of ibuprofen by *E. coli* epi300 pFOS3G7 despite the apparent presence of an invertase is somewhat puzzling; it may suggest that the true ibuprofen-CoA isomerase is present elsewhere on the fosmid or that inversion is either taking place abiotically or is being fortuitously catalyzed by an *E. coli* protein. Futher sequence analysis as expression will be required so as to more fully determine whether or not *ipfT* does indeed code for the first known bacterial 2-arylpropionyl-CoA, more specifically ibuprofen-CoA, isomerase. This gene product could be of industrial interest given the fact that the isomers of phenylacetyl-based NSAID drugs display remarkably different properties.

Conclusions

While the hypothetical missing gene was not found, several genes of interest were sequenced and characterized to some degree. The failure of the electron transport proteins *ipfI* and *ipfH* to complete the pathway along with pJ25 proved somewhat suprising and may indicate that pJ25 does not express one or more of the *ipfABDEF*

genes correctly. It may also indicate that *ipfIH* is not the electron transport chain of the *ipf* pathway but rather that the genes have yet to be located on pFOS3G7.

Initial data seems to indicate the IpfR plays a role in regulation of the *ipf* metabolic system on pFOS3G7 and pFOS4F6. Further sequencing and subcloning of the gene along with analyzing the different responses to substrate induction on the two fosmids and on *ipfR* mutants will further elucidate what role IpfR might play. Given that IpfR seems to be a repressor, shown by the fact that when it is disrupted by a transposon insertion, metabolic activity actually increases, clearly indicates that it is not required for metabolism and therefore not the elusive missing gene.

IpfT, which has similarity to an isomerase/transferase, may prove to be an ibuprofen isomerase. Further sequencing, subcloning and expression along with *ipfF* followed by enantiomeric analysis would reveal whether or not this is the case. Such an alpha-branched phenylacetate chiral isomerase would be the first such bacterial protein and could prove to be of commercial and industrial value.

Periplasmic binding protein IpfG

pFOS3G7Tn*:ipfG* was originally identified in the pFOS3G7 transposon library by its slower rate of catechol accumulation. HPLC analysis showed that ibuprofen disappearance was slower than *E. coli* epi300 pFOS3G7. The *ipfG* transposon mutant was sequenced using the transposon-specific primers FP1 and RP1 (Table 3.2) and characterized via blastx analysis (Altschul, Madden et al. 1997). and *ipfG* was hypothesized to code for the periplasmic binding component of an ABC transporter

system. Ibuprofen disappearance assay using the the methods for *E. coli* epi300 pFOS3G7 transposon mutants described in Chapter 3 revealed that *E. coli* epi300 pFOS3G7Tn*:ipfG* demonstrates reduced catechol production activity (46% of the activity of *E. coli* epi300 pFOS3G7, Figure A3.19). Uptake assays were performed on pFOS3G7Tn:*ipfG* using the methods described in Chapter 4. While for the most part uptake by pFOSTn:*ipfG* was no different than intact pFOS3G7, uptake by *E. coli* epi300 pFOS3G7Tn*:ipfG* was actually higher than *E. coli* epi300 pFOS3G7 at 2.5μM ($p<0.05$) (Figure A3.20).

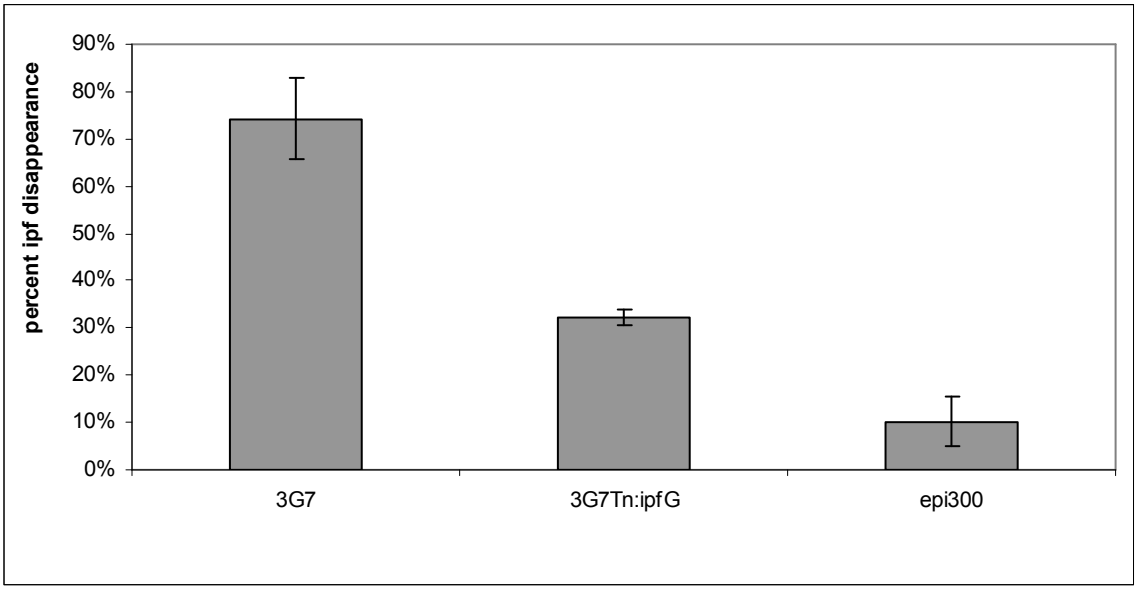


Figure A3.19. Disappearance of ibuprofen in *E. coli* epi300 pFOS3G7Tn:*ipfG* cultures compared to intact pFOS3G7 and vectorless epi300. 3G7tn:*ipfG* showed significantly reduced ibuprofen disappearance as determined by student's t-test.

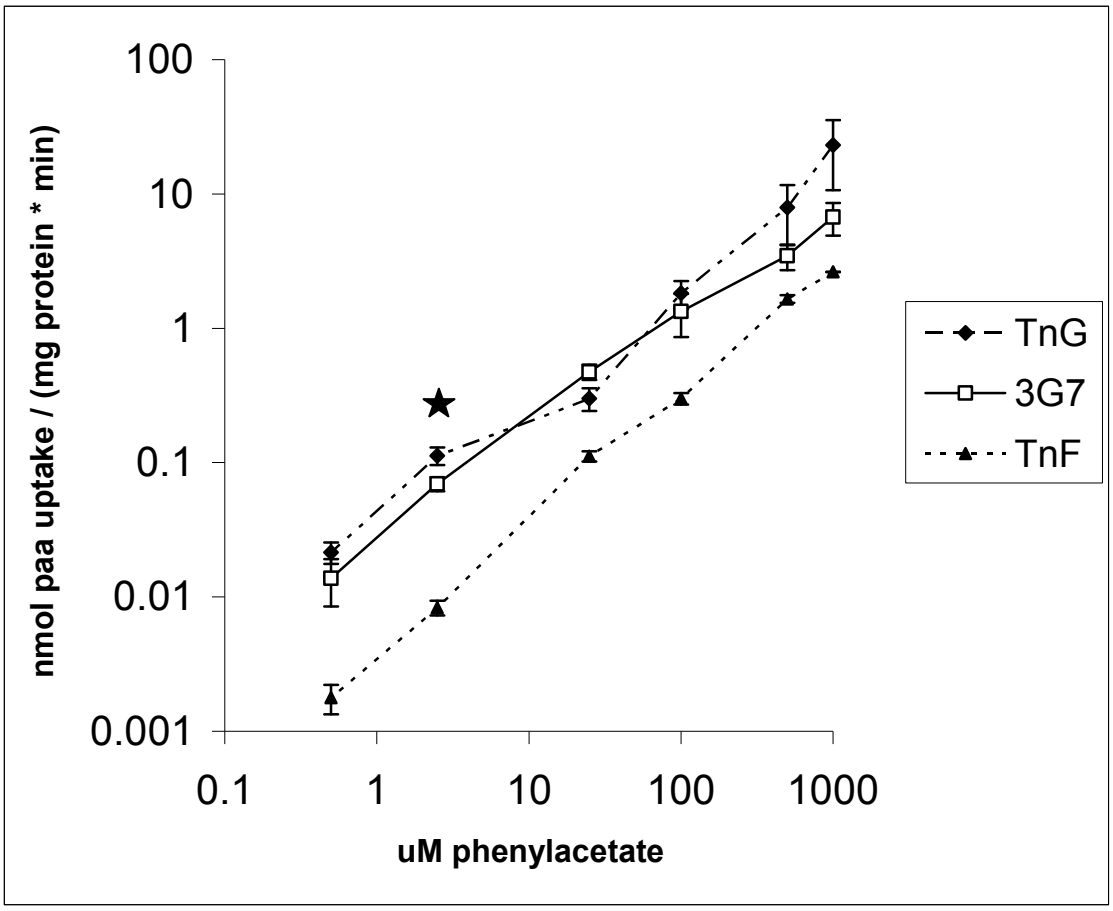


Figure A3.20. Uptake of radiolabelled phenylacetate by *E. coli* epi300 harboring pFOS3G7 or pFOS3G7 with transposon in *ipfG* or *ipfF*. Uptake by pFOS3G7Tn:*ipfG* was higher than pFOS3G7 at 2.5 uM phenylacetate as indicated by the star ($p<0.05$).

The role played by *ipfG* proved difficult to elucidate. While its interruption via transposon mutagenesis reduced the rate of ibuprofen metabolism, the same *ipfG* mutant showed no clear difference from the control strain in substrate uptake rate. However, any characterization of *ipfG* would have wide implications. Paul Black and colleagues in their pioneering work on fatty acid uptake mechanisms encountered a similar gene *tsp* (Azizan and Black 1994). The putative Tsp protein had similarity to periplasmic binding proteins of ABC transporter systems as well and was also found to adversely impact fatty acid metabolism through random transposon mutagenesis tests. However, the role that *tsp*

plays was likewise difficult to characterize and no work regarding it has been published since. It would be informative to confirm its projected localization in the periplasmic space and also to investigate its affinity for the substrate compound, perhaps by purifying large amounts of the protein and testing to see if radiolabelled substrate binds to the protein using western blotting and autoradiography. If localization to the periplasm and direct interaction of the protein with ibuprofen could be established, then the nature of its role in uptake could be infered. Perhaps such proteins are crucial for uptake under certain environmental or physiological states, such as particular substrate concentrations, stress levels, or under particular ionic or pH conditions. Given the occurrence of such proteins in bacteria and in animals, such work could have wide implications from both a purely scientific and an applied point of view

The predicted protein product of *ipfG* has high similarity to a variety of periplasmic binding proteins of ATP-binding cassette (ABC) transport systems. When *ipfG* was knocked out, metabolism of ibuprofen was reduced. The proximity of a putative ABC periplasmic binding protein to an aromatic acid CoA ligase is somewhat unexpected given that ABC transport systems and vectorial acylation have not been reported to be related to one another to any degree (Busch and Saier 2002). While ABC systems always have a membrane-spanning protein that serves to shuttle the chemical of interest across the membrane and an ATPase that powers the transport (Jones and George 2004; Szakács, Váradi et al. 2008) some, but not all ABC systems have periplasmic components (Jones and George 2004). There are very few examples, however, of the reverse: periplasmic binding proteins rarely occur without a cognate ATPase. The actual role played by periplasmic binding proteins is the subject of some debate. In fact, it has

been demonstrated that ABC systems that have an associated periplasmic binding protein do not require the periplasmic component for transport to function (Holland and Blight 1999). It has been suggested that the periplasmic protein merely serves to speed the diffusion of the target chemical across the periplasmic space to the inner membrane-spanning protein (Holland and Blight 1999).

This particular experiment was performed in order to determine if the product of *ipfG* was involved in uptake. ABC transporters are fully dependent upon the hydrolysis of ATP to drive transport. If both *ipfF* and *ipfG* were involved in uptake transposon inactivation of *ipfF* should have helped to reveal the role this putative ABC transport system was playing. The observation that the metabolic poisons, and thus a lack of available ATP or membrane charge, had no effect on uptake by the *ipfF* mutant suggests that the residual uptake was not dependent upon ATP or a chemiosmotic gradient, and thus was unlikely to be encoded by a standard ABC transporter such as *ipfG*.

In fosmid *E. coli* epi300 pFOS3G7 from Ibu-2, no ORFs have been located to date that correspond to the other component of an ABC system despite the fact that approximately 6kb of DNA has been sequenced in either direction of *ipfG*. While *ipfF* encodes ATP-dependent CoA ligase activity, and is therefore likely an ATPase, the ATP binding cassette of ABC systems is of a very specific tertiary structure that is characteristic of the group. IpfF shares no similarity with the ABC motif. The question stands then as to how ibuprofen is transported into the cell and what role, if any, does *ipfG* play in transport or metabolism of ibuprofen.

The clear inhibition of substrate disappearance (Figure 3.19) suggests a role in the pathway, though the uptake data showed that IpfG is not involved in transport. While

IpfG does share identity with characterized periplasmic binding proteins of ABC transporter systems, that similarity is low (20-25%). Additionally, Swiss-model was unable to build a three dimensional homology model with any level of confidence. Taken together, the paucity of information regarding IpfG-like proteins leaves wide-open what role it might play in metabolism. Given that there over 100 putative genes with over 40% identity in the NCBI database, none of which has been characterized to any degree, further investigation of the role of IpfG may prove valuable to the scientific community.

In the case of bacterial fatty acid metabolism, what happens to the substrate once it reaches the periplasmic space is poorly understood. It seems that the high hydrophobicity would discourage the fatty acid's movement across the aqueous periplasmic space, a situation exacerbated by the acidic nature of the periplasmic space which would serve to protonate fatty acids, making them even more hydrophobic. A gene named *tsp* has been shown to facilitate, but not be necessary for fatty acid transport in *E. coli* (Azizan and Black 1994). The gene product is water soluble and localized to the periplasmic space, but little else is known about it. *ipfG* is somewhat similar to *tsp* (21% identity / 34% similarity), with analogous residues distributed across the entire length of the sequences. The protein *tsp* was shown in one case (Azizan and Black 1994) to speed fatty-acid transport but has not been the subject of any published research since. The most similar protein in the Swiss-prot database was BraC, the periplasmic binding component of a branched-chain amino acid ABC transport system in *P. putida* (Hoshino and Kose 1990), with which IpfG shares 25% identity and 46% similarity.

Schuehle *et al.* (Schuehle, Jahn et al. 2001) described a similar situation to that presented by *ipfFG* in Ibu-2: in *Azoarcus evansii,* a 2-aminobenzoate CoA ligase is located adjacent to a putative periplasmic binding protein in the same gene order as that of *ipfFG* (42% and 39% similarity respectively). The role of the periplasmic binder has not been investigated in this system, but this example suggests that the co-localization of these two types of genes may not be an accident, but rather suggests that the IpfG-like protein might play some enigmatic role in the transport system.

In this system IpfG actually seemed to be working against the uptake process at 2.5 μM, leading to the possibility that IpfG might actually play a role in excretion at low phenyacetate concentrations (<50 uM). However, these should be regarded as preliminary results the role they play in the transport process is still unclear.

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APPENDIX 4

ADDITIONAL EXPERIMENTS AND LINES OF INQUIRY

Preface

Herein are described several experiments and pilot projects related to the Ibu-2 project. The rationales, methods, and results are presented in abbreviated form.

Screening, Metabolic, and Genetic Work done prior to the Fosmid library

Screening assays

Two colorimetric indicators had been shown to be useful in the detection of *ipf* pathway intermediates; catechol polymerization which is possible to enhance by addition of ferric chloride and p-toluidine and the yellow color consistent with metacleavage product of catechols. Several additional chemicals were investigated for utility as chromogenic reagents indicative of *ipf* pathway activity or the presence of *ipf* pathway metabolites.

Napthalene dioxygenase acts on indole to create blue indigo (Ensley, Ratzkin et al. 1983) while benzoate dioxygenases are able to act on indolecarboxylic acids to produce pigments (Keener, Watwood et al. 2001). Ibu-2 was grown on ibuprofen in the presence of these chemicals (indole, indole-2-carboxylic acid, indole-3-carboxylic acid)

at two concentrations in the case of the indolecarboxylic acids (150 and 600ppm) with no detectable color change. Pseudomonas HK44, a naphthalene degrader, was used as a positive control for color generation for indole.

Next, indoleacetic acid was screened for activity under the hypothesis that the *ipf* pathway might be able to act upon this aromatic acetic acid. As no such activity has ever been demonstrated, no positive control was available. A variety of indoleacetic acid concentrations were tested (200, 300, 400, 500ppm). No ibuprofen-dependent color change was detected in Ibu-2 cultures.

The nitro blue tetrazolium / 2,3,5-triphenyl-2H-tetrazolium chloride (NBT/TTC, 20ppm/25ppm) indicator described by Finette *et al.* (Finette, Subramanian et al. 1984) for the detection of toluene dioxygenase electron transport chain activity was also employed. The NBT/TTC indicator did not discriminate between ibuprofen grown and glucose grown Ibu-2. All colonies turned slightly reddish-brown with no blue color indicative of NBT reduction by a ferredoxin reductase.

Mineralization assays

In order to confirm the mineralization of ibuprofen and to gain insight into the fate of the carboxyl group, ^{14}C-ibuprofen, labeled only on the carboxyl group, was used in classic mineralization assays. A 5% inoculation of exponential phase Ibu-2 was added to 5ml of 500ppm ibuprofen MSM media in a 40ml conical vial that had been spiked with 50,000 dpm of ^{14}C-ibuprofen. A small vial of 1ml 1M NaOH was placed in each sample. The NaOH vial serves as a CO_2 trap. The NaOH trap was removed and replaced

periodically. Total dpm per time point was measured by scintillation counter (Figure A4.1).

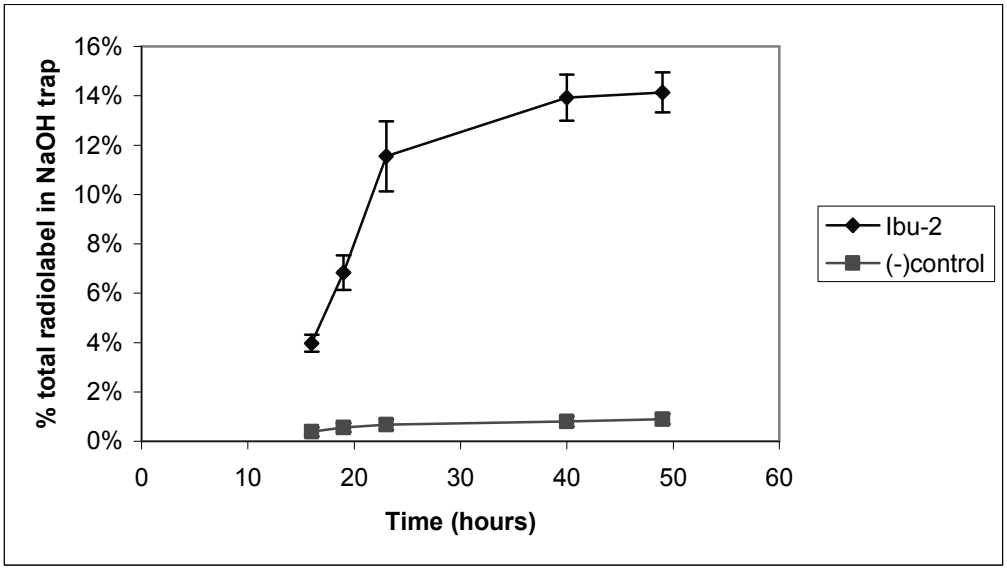

Figure A4.1. The percent of total radiolabel present in sodium hydroxide trap following growth of Ibu-2 in 500ppm ibuprofen MSM in the presence of 50,000 dpm ^{14}C-ibuprofen. This experiment was performed in plastic culture tubes.

The data demonstrated that Ibu-2 was mineralizing ibuprofen, but only 14% of the radiolabel ended up in the NaOH trap, leading to the assumption that the remainder had either been incorporated into biomass or remained in the media in some form, either as unmetabolized substrate or as a waste product. However, measurement of the radioactivity present in the total active culture samples revealed only 5.1% (+/- 2.2%) in the biomass and 1.9% (+/- 0.2%) in the media, leaving a total of 79% of the radiolabel unaccounted for. On the other hand, the negative control media still contained 65% (+/- 12%) of the radiolabel. In other words, the active culture was missing 44% of the total radiolabel when compared to the negative control. Two hypotheses for where the missing radiolabel might have gone to, CO_2 that escaped the reaction chamber or a waste product that sorbed to the plastic culture vial, were addressed in a following experiment.

In order to prevent possible escape of radiolabelled CO_2 via a poorly sealed reaction chamber, the 40ml plastic conical vials were replaced with 40ml glass vials, the lids of which had a soft plastic seal. The use of glass vials instead of plastic also addressed the possibility that some radiolabel was being tied up in a waste product with affinity for the plastic vial walls. Furthermore a chloroform extraction of the glass tubes upon termination of the assay in order to remove possible hydrophobic insoluble or glass-sorbing radiolabel. The amount accumulating in the NaOH trap in the Ibu-2 samples (14.2% +/-3.3%) was almost identical to the previous value (Figure A4.2).

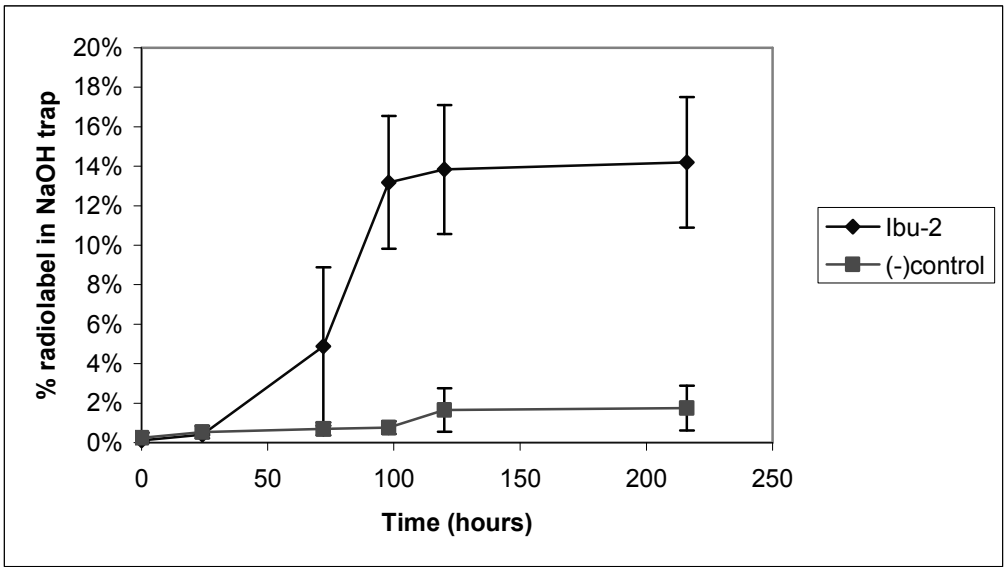

Figure A4.2. The percent of total radiolabel present in sodium hydroxide trap following growth of Ibu-2 in 500ppm ibuprofen MSM in the presence of 50,000 dpm ^{14}C-ibuprofen. This experiment was performed in rubber-sealed glass tubes.

The culture and media of the Ibu-2 samples contained only 11.4% (+/- 6.8%) of the total radioactivity added while the negative control media contained 80% (+/- 0.7%). The choloroform extracts of the culture vials contained no measurable radioactivity, indicating that all of the residual media radiolabel was water soluble. Again, most of the

radiolabel that was added to the Ibu-2 cultures, 57%, was unaccounted for when compared to the negative control.

Both experimental systems resulted in almost identical trapping of radiolabel (14.1% vs. 14.2%), though there were differences in the amount of missing radiolabel versus the negative control; 44% for the plastic vial versus 57% for the glass. One explanation might be that the periodic opening of the vials led to release of radiolabel that had for one reason or another had not partitioned into the NaOH trap. The NaOH trap was tested at termination of the assay to confirm that it was still strongly alkaline, which it was. The remarkable difference in missing radiolabel between the Ibu-2 samples and the negative controls indicates that the loss was biologically mediated in some way; perhaps there was a volatile radiolabelled waste product that did not readily partition into the NaOH trap.

It is interesting to note that the negative controls were also missing notable amounts of radiolabel, 35% for the plastic and 20% for the glass, which seems to indicate that there was a consistent leakage or measuring error in at least one point in the system. The loss of radiolabelled ibuprofen from the negative control might be due to volatilization of ibuprofen and loss via the gas phase and is somewhat consistent with the hypothesis that the Ibu-2 cultures were releasing a volatile radiolabelled waste product.

Investigation of megaplasmids in Sphingomonas Ibu-2

Sphingomonas aromaticivorans F199 (Romine, Stillwell et al. 1999) has a 184 kilobase megaplasmid that harbors several aromatic catabolic clusters. In order to address the possibility that Ibu-2 harbors the *ipf* pathway genes on a similar

megaplasmid, a two tiered approach was employed. Firstly, Ibu-2 total DNA was subjected to pulsed field gel electrophoresis (PFGE), which separates chromosomal DNA from large plasmid DNA. Three to nine possible megaplasmids of indeterminate size were observed (Figure A4.3).

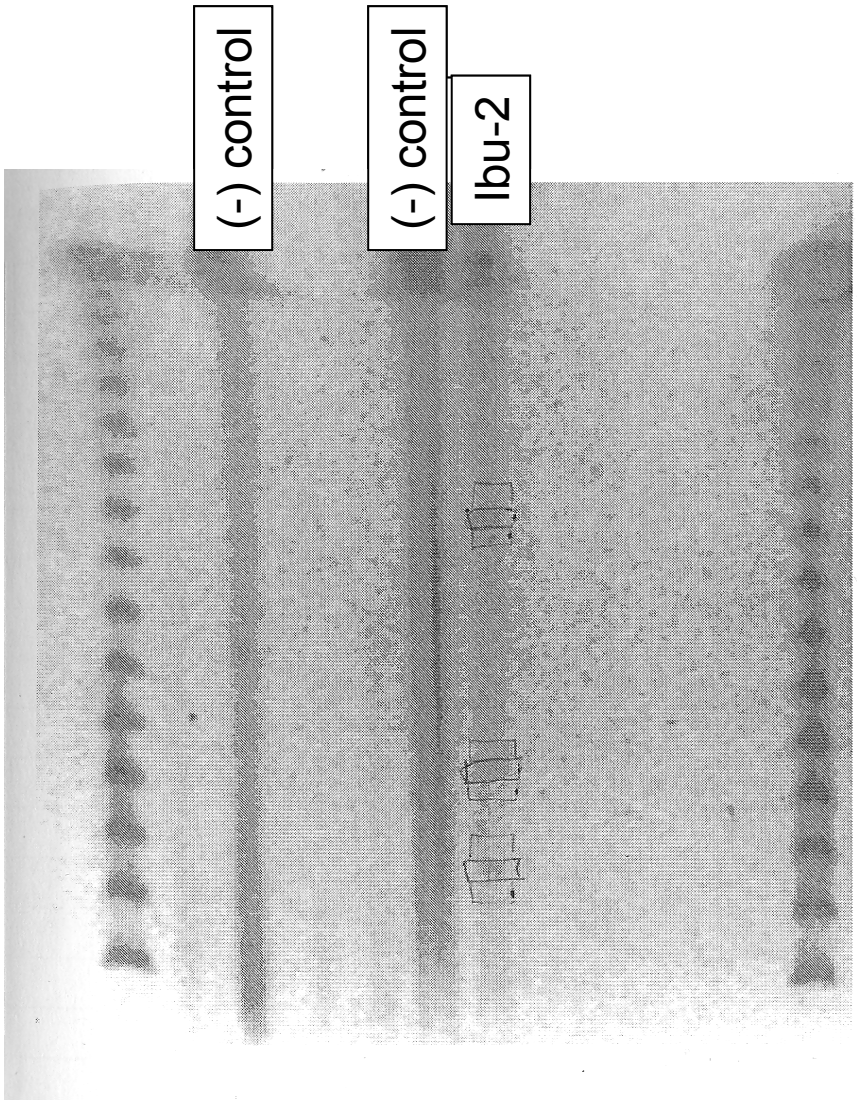

Figure A4.3. PFGE of Ibu-2 total DNA (third lane) with two non-megaplasmid containing *E. coli* controls in the first two lanes. Faint bands possibly representing megaplasmids are outlined.

Each of these bands was cut out, subjected to three freeze thaw cycles, and centrifuged for fifteen minutes at 15,000 G in order to remove them from the agarose matrix. Each sample was then subjected to PCR analysis with the dmpB and dmpC primers (Table A4.2, Figure A4.4).

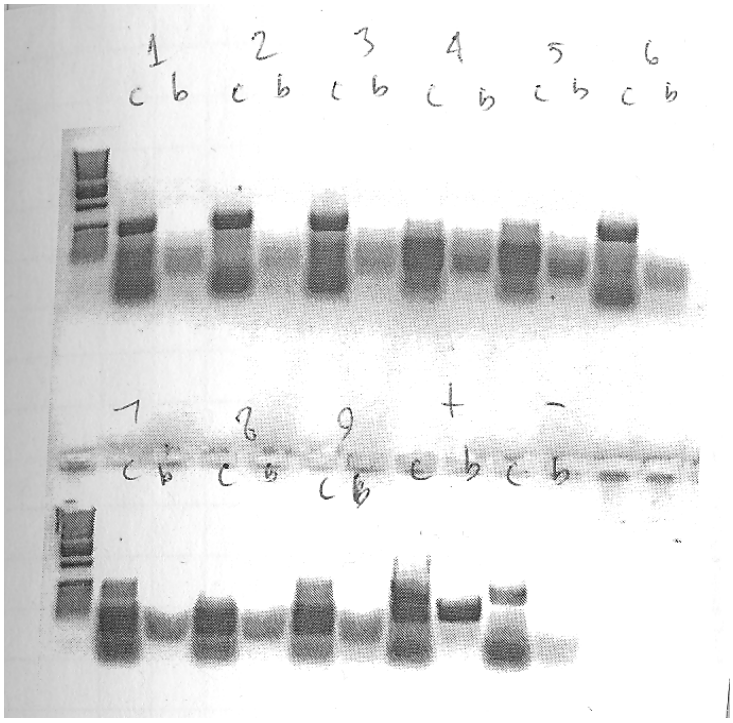

Figure A4.4. Gel electrophoresis of PCR screening reactions of the nine putative megaplasmid gel purifications. "1" employed the largest band as template, "2" the next largest and so forth. "+" is positive control reactions (diluted Ibu-2 PCR products) and "-" is non-template controls. "c" is dmpC primed reactions while "b" is dmpB primed reactions.

These results suggested that, assuming that each triad of bands represented a single megaplasmid in three different secondary structures, the largest of the megaplasmids had a *dmpC*-like catechol metacleavage gene.

In order to investigate whether or not the megaplasmids are necessary for growth of Ibu-2 on ibuprofen, Ibu-2 was subjected to plasmid curing via two methods. Firstly a method adapted from Crosa *et al.* (Crosa and Falkow 1981) in which Ibu-2 was streaked

on 1/10 LB plates in the presence of a range of acradine orange concentrations (75, 100, 125, 150, 175, 200, and 600 ppm). Acradine orange inhibits DNA replication and selectively inhibits the replication of plasmids. Each resulting treatment was still able to grow on ibuprofen as sole carbon and energy source. Second, Ibu-2 was streaked six successive times on 0.2% glycerol MSM plates on the assumption that after so many generations in the absence of selective pressure, any specialized catabolic plasmid would be lost. Ibu-2 remained able to grow on ibuprofen as sole carbon and energy source after this treatment.

While it did appear that Ibu-2 carries up to three megaplasmids and that one or two of them have genes associated with aromatic metabolism, neither plasmid curing methods led to loss of ability to grow in ibuprofen.

Sphingomonas Ibu-2 antibiotic resistances and attempts to create knockouts

Ibu-2 was screened for antibiotic resistances by plating on LB solid media with the presence of standard concentrations of ampicillin, kanamycin, tetracycline, and rifampicin. Ibu-2 was able to grow in the presence of ampicillin and rifampicin, but not tetracycline or kanamycin.

In order to create a strain of Ibu-2 less prone to contamination during long growth incubations, plasmid pBBR1mcs-3, which carries a tetracycline resistance marker, was cloned in via triparental mating with *E. coli* S17 pBBR1mcs-3 and *E. coli* BH101 pRK2013, a helper plasmid for the transmission of non-transmissable plasmids (Vallaeys, Albino et al. 1998). Negative selection against the *E. coli* strains was achieved by plating

the mating on minimal media. Mating success was confirmed by plasmid purification. *Sphingomonas* Ibu-2 pBBR1mcs-3 was placed in the culture collection.

An attempt was made to construct an Ibu-2 transposon library using the plasposon pTn*Mod*OTC (Dennis and Zylstra 1998) was unsuccessful as no tetracycline resistant tranposon mutants were recovered from transformation of chemically competent Ibu-2.

Additionally, an Ibu-2 dmpC PCR fragment was cloned into the suicide vector pLD55. The resulting vector was maintained in *E. coli* BW21037 and an attempt to clone into Ibu-2 via triparental mating with *E. coli* HB101 pRK2013 was unsuccessful.

Generating an isobutylcatechol standard

An attempt to create large quantities of isobutylcatechol, which is not commercially available, was undertaken by incubating *E. coli* JM109 (DE3) pDTG141 (Gibson, Resnick et al. 1995) with isobutylbenzene. JM109 (DE3) pDTG141 has the capacity to deoxygenate naphthalene and indole. The strain was incubated with 50ppm isobutylbenzene in LB media with 100ppm p-toluidine and 1.5mM ferric chloride to detect any accumulating catechols. No catechols were detected.

There are also three point mutations in pDTG141 that alter substrate specificity (Gibson, Resnick et al. 1995). These constructs might prove useful for generating isobutylcatechol, but were not screened for activity.

Early attempts to gain a foothold in the Ibu-2 ibuprofen catabolic genes

Prior to the creation of the fosmid library as described in Chapter 3, attempts to identify *Sphingomonas* Ibu-2 genes involved in the metabolism of ibuprofen revolved

around the use of degenerate primers for the amplification of general aromatic-type genes; the dmpC primers amplify a small conserved fragment semimuconic acid (meta cleavage product) dehydrogenase, while the dmpB primers amplify an internal fragment of catechol 2,3 dioxygenase meta-cleavage genes. Both primer sets yielded fragments of expected size from Ibu-2 (Figure A4.5). Two dmpB amplicons and a dmpC amplicon were cloned into pGEMt-easy. The dmpB amplicon was sequenced and specific primers named "Ibu2cat23" F/R were designed. Sequences of these fragments were consistent with the putative gene identity. This project was later abandoned in light of the rich data provided by the fosmid library; the amplicons were never compared to Ibu-2 fosmid library sequences.

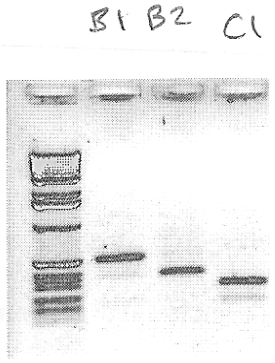

Figure A4.5 Purified PCR products using the dmpB and dmpC primer sets on Ibu-2.

In order to determine whether the *dmpB*-like gene was upregulated by, and therefore presumably involved in, ibuprofen metabolism, an RNA dot-blot was performed. RNA from LB and ibuprofen grown Ibu-2 cultures was isolated and spotted onto membranes. The membrane was probed with P^{32}-labelled Ibu2cat23 amplicon. The results were somewhat suggestive of Ibu2cat23 up-regulation but were unclear, with only

one of three triplicate spots of RNA from ibuprofen-grown Ibu-2 showing hybridization (Figure A4.6)

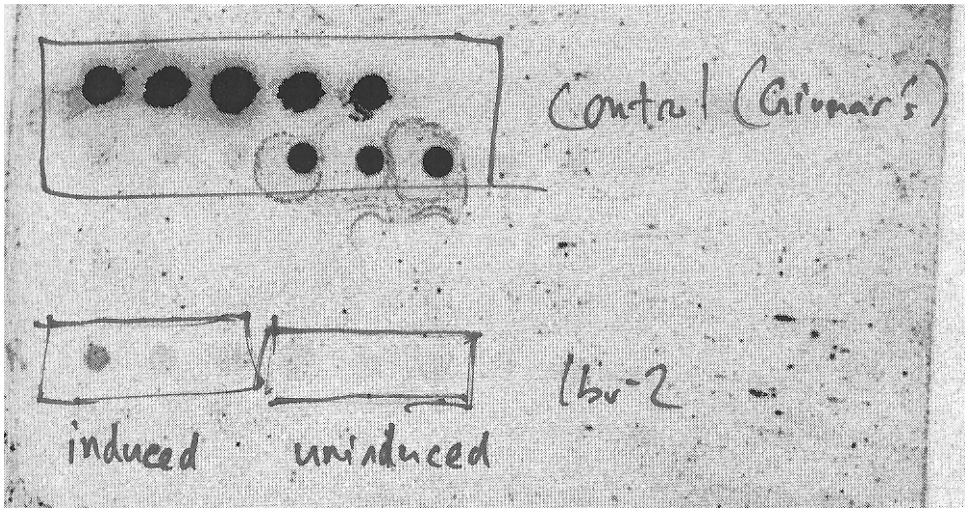

Figure A4.6. RNA dot blot comparing induced and uninduced Ibu-2 probed with ibu2cat23 radiolabeled PCR product.

The primers were not specific enough so as to yield any useful information via sequencing directly from Ibu-2 DNA. An attempt to obtain additional larger fragments by PCR amplification using one dmp primer and a random hexamer was unsuccessful. Because direct sequencing approaches were fruitless, digestion/probing methodologies were employed.

Firstly Ibu-2 DNA was digested with a concentration of Sau3AI that methodological development assays had shown would yield an average of 10kb fragments (Figure A4.7). The digested DNA was cloned into pBBR1mcs-3 and cloned into *E. coli,* resulting in an approximately 5000 clone a chromosomal library. The library was transferred to membranes and probed with a P^{32}-labelled Ibu2cat23 amplicon. The probing identified six clones (Figure A4.8). The plasmids from the six dmpC-positive library clones were isolated, but determination of their sizes revealed only 1-2kb insert

size. PCR with both the Ibu2cat23 and dmpC primer sets revealed that each clone had both genes (Figure A4.9) and sequencing with Ibu2cat23F/dmpCR revealed that the two genes were adjacent to one another in each case (Figure A4.10). A sequencing project was undertaken but put on hold in lieu of fosmid library generation.

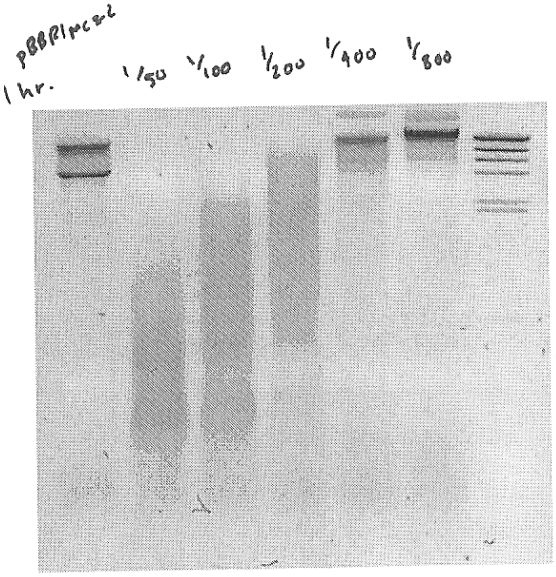

Figure A4.7. Dilution restriction enzyme method development. Fraction of one unit of SmaI (shown above the lanes) incubated with Ibu-2 DNA for four hours.

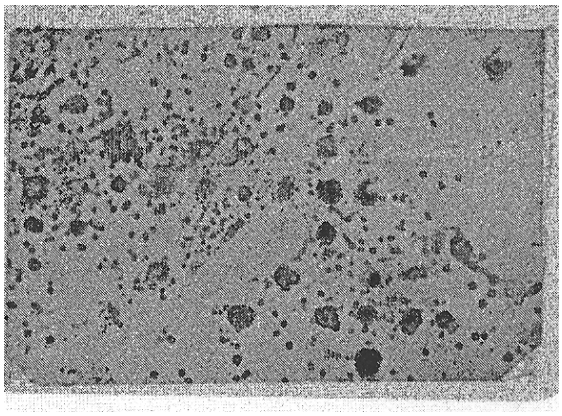

Figure A4.8. Representative pBBR1mcs-3 Ibu-2 chromosomal library probed with ibu2cat23 radiolabelled PCR product. Positive hybridization is at the bottom of the membrane.

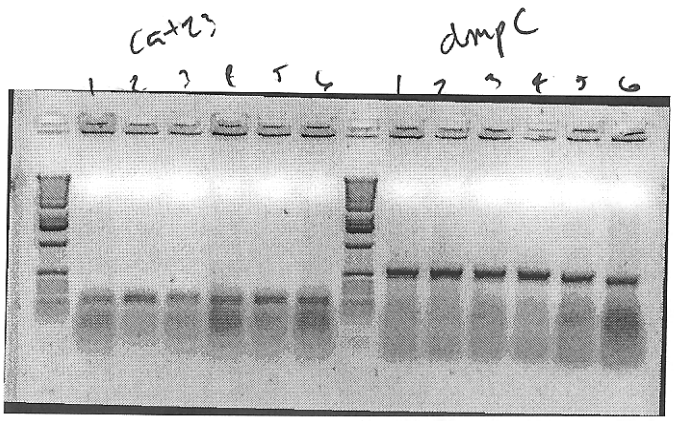

Figure A4.9. ibu2cat23 and dmpC primed PCR reactions using the six ibu2cat23 positive pBBR1mcs-3 Ibu-2 DNA chromosomal library clones. Products match the expected sizes.

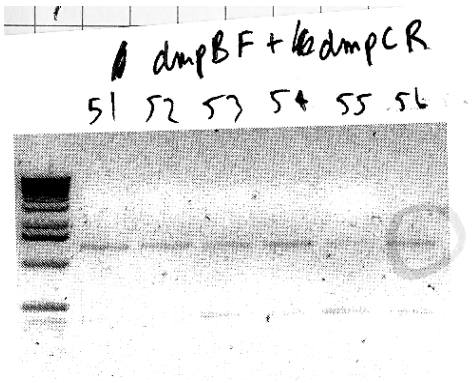

Figure A4.10. PCR products using ibu2cat23F and dmpCR primers on the six ibu2cat23 positive chromosomal library clones. The amplicon is approximately 1400bp.

Second, *Sphingomonas* Ibu-2 chromosomal DNA was isolated and overdigested with EcoRI, SmaI, and BamHI alone and in combinations, separated by gel electrophoresis, and transferred to a membrane (Figure A4.11a). The membrane was probed with a P^{32}-labelled Ibu2cat23 amplicon (Figure A4.11b). A project was undertaken to clone a 2kb EcoRI / SmaI fragment into a pBBR1mcs vector but put on hold for the generation of the fosmid library.

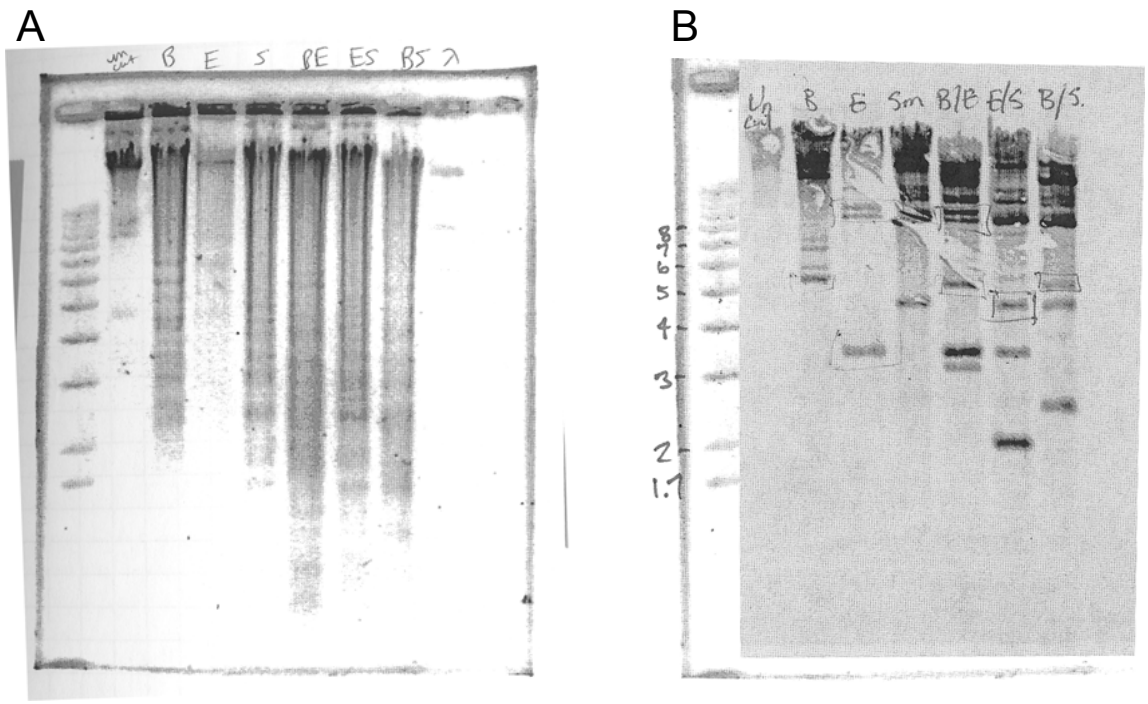

Figure A4.11. B; BamHI, E; EcoRI, S; SmaI. A: Overnight overdigestion of Ibu-2 chromosomal DNA using the enzymes indicated above the lanes. B: Result of probing with radiolabelled Ibu2cat23 PCR product.

Activity of ibuprofen-induced aerobically grown Ibu-2 under anoxic conditions

Given that coenzyme A ligase mediated aromatic acid metabolic pathways have been more commonly associated with anaerobic metabolism, a small experiment to demonstrate the oxygen-requirement of the capacity for aerobically ibuprofen grown *Sphingomonas* Ibu-2 to degrade ibuprofen was designed and executed.

Sealed 10ml glass bottles were filled with 2ml of concentrated washed resting ibuprofen-grown Ibu-2. Nitrogen gas was bubbled through three samples for thirty minutes, and three were left undisturbed. 200ppm of ibuprofen was added from a 1% methanol stock solution and the samples were shaken for two hours. The samples were killed with 0.1% sodium azide and samples prepared for ibuprofen quantification via HPLC.

During the two hour incubation, the aerobic samples turned yellow in a manner consistent with metabolism and accumulation of meta-cleavage product. In the aerobic samples, 42.4% (+/- 5.8%) of the ibuprofen remained while in the anaerobic samples, 98% (+/- 2%) remained. These results are consistent with the oxygen requirement of the *ipf* catabolon and support the involvement of the putative IpfAB dioxygenase in the pathway. If Ibu-2 does possess the ability to metabolize ibuprofen under anaerobic conditions, which was never tested, it would seem that any such pathway is active only when induced. The presence of an anaerobic pathway is somewhat suggested by the utilization of a coenzyme A ligase (IpfF), which has been hypothesized to be employed particularly in facultative anaerobic bacteria as a universal dual-purpose reaction intermediate in terms of general metabolic strategy (Fuchs 2008).

Constructs and Cell-Free Assays

Meta-cleavage product degradation assay

Ibu-2 has been shown to grow on the methylated phenylacetic acids 4-tolylacetic acid (4TAA), 3-tolylacetic acid (3TAA), and (2-(4-tolyl)propionic acid (24TPA), but not on the non-ring substituted phenylacetic acid (PAA) and 2-phenylpropionic acid (2PPA) (Chapter 2). Additionally, it has been shown that the two non-growth substrates were metabolized via the same catechol intermediate. One possible explanation of the failure of growth would be that the meta cleavage product (mcp) of catechol is not metabolizable. The first step in metabolism of mcp is typically via an NAD+ dependent

dehydrogenation of the terminal aldehyde group; indeed, the product of this reaction was detected via GC/MS of Ibu-2 culture supernatant (Chapter 2).

In order to investigate whether or not such a dehydrogenase was at work, cell-free extract was incubated with fresh mcp with and without the addition of NAD+ and the rate of disappearance of the mcp was measured via kinetic spectophotometric reading. Firstly, mcp was generated by ibuprofen-grown resting 40-fold concentrated Ibu-2 preparations by adding 5 mM of either catechol, 3-methylcatechol, or 4-methylcatechol for 30 minutes. The supernatant was removed and the mcp characterized via UV-vis spectral analysis (figure A4.12).

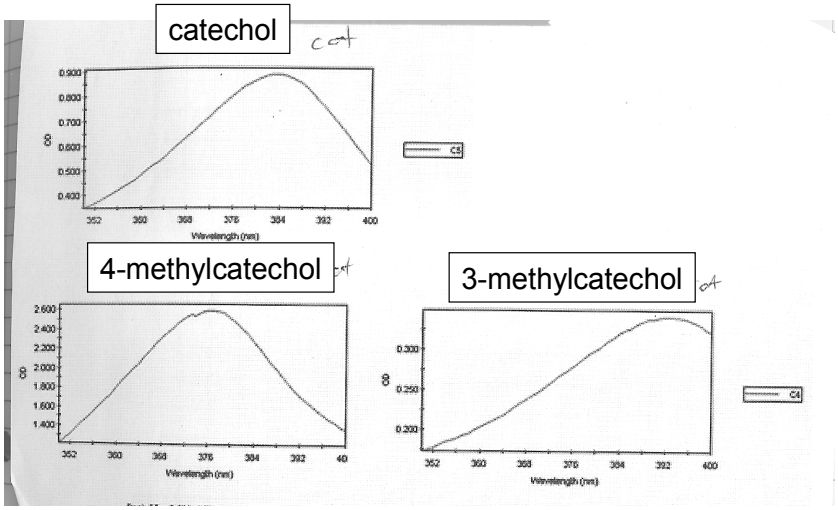

Figure A4.12. UV-vis spectrum (350-400nm) of the supernatants of Ibu-2 resting cell assays exposed to catechol, 4-methylcatechol, or 3-methylcatechol.

The mcp preparation was then combined with cell free extract from ibuprofen-grown Ibu-2 with and without the addition of 5mM NAD+. A negative control without addition of cell-free extract was also included. The samples, prepared in triplicate, were immediately placed in a KCjunior plate reader for kinetic measurement of absorbance at 382nm, a

wavelength at which all three mcps absorb strongly. The disappearance of the mcp over 15 minutes was plotted and a slope calculated.

Table A4.1. The rate of the disappearance of metacleavage product of the indicated chemical when exposed to Ibu-2 cell free extract and NAD+.

	Rate of dissappearance of mcp, OD382/min. (sta		
meta-cleavage product of:	extract	extract plus NAD+	NAD+
catechol	-4.09 (1.11)	-25.55 (0.94)	-8.2 (0.17)
4-methylcatechol	-10.6 (2.3)	-21.50 (6.24)	-3.32 (0.29)
3-methylcatechol	-19.68 (3.12)	-17.98 (6.84)	-1.69 (0.15)

The disappearances of catechol mcp (5-fold increase, $p<0.05$) and 4-methylcatechol mcp (2-fold increase, $p<0.05$) were both strongly enhanced by the addition of NAD+ while the disappearance of the mcp of 3-methylcatechol was not affected by NAD+ addition (table A4.1).

While the results did not confirm the hypothesis that Ibu-2 fails to grow on PAA and 2PPA due to a dead-end mcp product of catechol, the results were fully consistent with what is generally known regarding lower pathways and ring substitutions (figure A4.13).

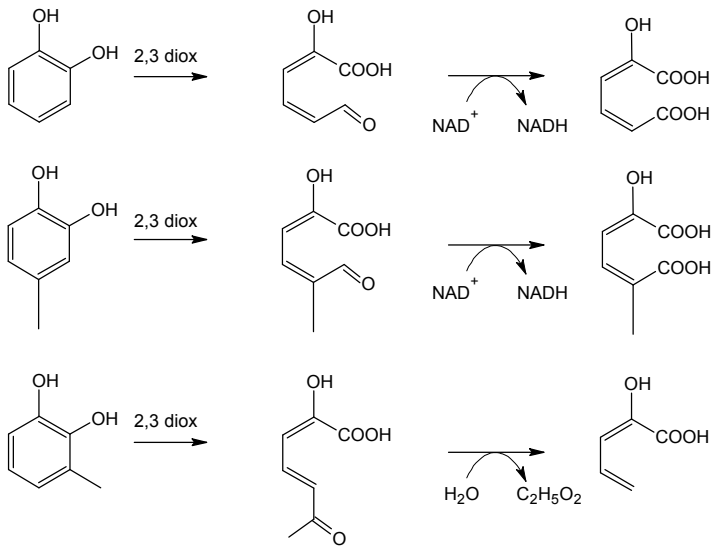

Figure A4.13. Widely distributed bacterial strategy for the metabolism of metacleavage products of catechol (top), 4-methylcatechol (middle), and 3-methylcatechol (bottom) (Murray, Dugoleby et al. 1972).

In many lower pathways studied to date (Murray, Dugoleby et al. 1972) employ two different partial pathways depending on the ring substitution. Notably, while non-substituted and 4-substituted catechols tend to be dehydrogenated with NAD+ following ring cleavage, 3-substituted catechols are instead subjected to non-NAD+ dependent hydrolysis.

Catechol 2,3 dioxygenase

As described elsewhere in this section, a catechol 2,3 dioxygenase was identified during early degenerate primer PCR on *Sphingomonas* Ibu-2. During analysis of fosmid transposon libraries as described in Chapter 3, additional sequencing on non loss of function mutants described the DNA sequence upstream of *ipfA* (figure A4.14). Immediately upstream of *ipfA,* an ORF with high similarity to catechol 2,3 dioxygenases was identified and cloned into pGEMt-easy. The *E. coli* JM109 pGEM:Ibu2cat23 clone proved to readily degrade catechol to ring cleavage product (Figure A4.15). The

Ibu2cat23 ORF has a strong conserved ribosomal binding site seven bases upstream of the putative start codon (Figure A4.16).

Partial evidence of at least one other catechol 2,3 dioxygenase gene was found on other locations of pFOS3G7 and pFOS4F6. The sequence information can be found in the GeneQuest sequence assembly projects on the attached data DVD.

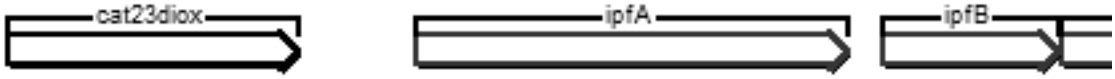

Figure A4.14. Location of the putative catechol 2,3 dioxygenase relative to *ipfA* and *ipfB*.

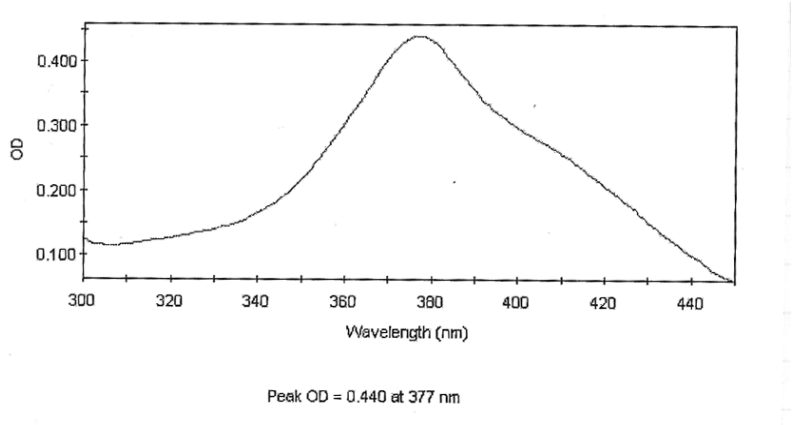

Figure A4.15. UV-vis spectrum of the supernatant of *E. coli* JM109 pGEM:Ibu2cat23 with 500ppm catechol. Maximum optical density = 377nm.

TCGAATGTCGATTATATTATCGAGATTATGACGAGACACACGTTGCTGAGCCTTGAT
TCGGTCCATGCTGAAGTGTGGCGAAATCTTCTGTAATGACCAAGTGAAAAATACAAT
GAATGCGAGAACTTCAAA**ATGAGTATCAAAAGCTTGGGTTACATGGGATTCGCGGTC**
AAGGATGTGCCCGCCTGGCGTTCTTTCCTGGCCCAGAAATTGGGCTTGATGGAAGCA
GGCGTCACCGATGACGGCGCCTTGTTTCGCATCGATTCGCGCGCCTGGCGGATTGCC
GTCCAGCAGGGCGAGGCAGACGATTTGGCCTACGCTGGCTACGAGGTGGCCGATGCA
GCGGGTCTGACGCAGATGACAGAAAAACTGCGGCAGGCCGGAATCGAAGTGGTCACG
GGCGATGTCGAACTGGCCAAGCGGCGCGGCGTGATGGGCTTGATTTCGTTTGCCGAT
CCGTTTGGTCTGCCACTGGAAATTTATTATGGCGCCAGCGAGGTGTTCGAAAAACCG
TTCATGCCCGGCGCGGCGGTGTCGGGTTTTCTGACCGGCGAGCAGGGGCTGGGTCAT
TTTGTGCGCTGCGTGCCGGATTCGGACAAGGCGCTGGCGTTTTATACCCAGGTGCTC
GGCTTCCAGTTGTCGGACGTCATCGACATGAAAATGGGACCGGACGTGACGATTCCG
GCGTATTTTCTGCACTGTAACGAACGCCACCACACCCTCGCGATCGCAGCTTTTCCG
CTGCCCAAGCGCATTCACCACTTCATGCTCGAAGTCTCGTCGCTCAATGACGTCGGT
TTTGCGTTTGACCGGGTTGACGCTGACGGCTTGATCACCTCCACGCTGGGGTGCCAC
ACCAATGACCATATGGTGTCGTTCTATGCAGCGACCCCGTCCGGAGTCGAGGTCGAG
TACGGCTGGGGCGCCCGCACCGTTGACCGCTCCTGGGTGGTGGCACGGCACGATAGC
CCGAGCATGTGGGGCCATAAGTCGGTGCGCAACAAAGCATAAACAACAAGCTGACCC
ACACAGGAGTTTTTGAATGAAGCTTTACTACAGCCCTGGCGCCTGCTCCCTGTCCCC
GCACATCGCCTTGCGCGAGGCGGGCCTGAACTTCGAGCTGGTACAAGTCGATCTGAC
AT

Figure A4.16. Sequence of Ibu-2 ORF (shown in bold type) consistent with catechol 2,3 dioxygenase. Putative ribosomal binding site (underlined).

Phenylacetyl-CoA disappearance when incubated with Ibu-2 cell-free extract

Partially in the hopes of identifying novel reaction intermediates, ibuprofen grown Ibu-2 cell free extract was incubated with 3mM phenylacetyl-CoA (PAACoA) with the addition of 3mM NADH. For this pilot assay, a no-enzyme treatment was used as a negative control. The general cell-free protocols described in Chapter 4 were employed. PAACoA was monitored via HPLC, which was performed using 84% 30mM phosphate buffer pH 7.4, 7% acetonitrile, 9% methanol running buffer with detection at 254nm. After two hours of incubation, 69% (+/- 1.5%) of the PAACoA was absent from the samples while none had degraded in the no-enzyme control. No PAA was detected via HPLC, indicating that the PAACoA disappearance was not simply due to hydrolysis of

the PAA-CoA thiol bond. However, no notable novel peaks were detected in this HPLC analysis.

While this line of research suggested that cell-free extract has the capacity to degrade PAA-CoA to an unknown product, it was not investigated further. An investigation of cofactor requirement, inducibility, or the utilization of denatured cell-free extract might have led to insights regarding whether the PAA-CoA disappearance was actually being catalyzed by the *ipf* catabolon.

Cloning ipfFG

Briefly, *ipfF* and *ipfG* were cloned adjacently by pGEMt-easy ligation to the PCR amplicon of ipfFF/ipfGR primers. The size and orientation of the construct were confirmed by PCR of the construct (successful amplification with M13R/GR primers). A successful construct was stored in the culture collection.

Cloning the ferredoxins

Two ORFs with similarity to ferredoxin encoding genes were found during the fosmid sequence assembly project. One, a plant-like ferredoxin encoding ORF, was described in Appendix 3. The other was found 3600 bp downstream of *ipfF*. Cloning primers were generated for both (Table A4.2) and both were cloned into pGEMt-easy (and stored in the culture collection) (Figure A4.17). While directional PCR screening revealed that the insert in both constructs was in the reverse orientation in respect to the *lac* promoter, the plant-like ferredoxin (*ipfI*) turned red, suggesting the presence of a cloned promoter region.

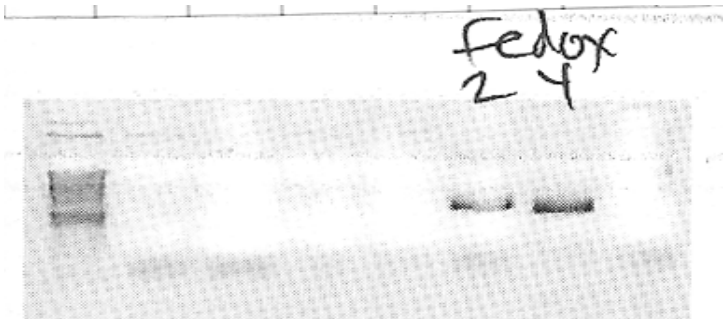

Figure A4.17. T7F / gene-specificR primed PCR product of pGEMt-easy ferredoxin constructs.

Table A4.2. Sequences of primers described in this appendix.

primer name	sequence
dmpBF	CGACCTGATCTCCATGACCGA
dmpBR	TCAGGTCAGCACGGTCA
dmpC333F	CCGCACGTTTCGACGAGTTCC
dmpC890R	AGACGATGCCGGCGTTCTTGC
ibu2cat23F	CCTGATCTCCATGACCGA
ibu2cat23R	ACGGTCAGGAAACGTTCG
ipfFeDox2F	AGCAGGAATATTACAGCCGCGACA
ipfFeDox2R	AGTCCTCAACCTCCTGGTGAAGAT
ipfFeDox4F	GCGAGATCCCGGATGCGAATTT
ipfFeDox4R	ACCGGATAGGCACGCGTTATTGAT

In the hope of identifying fosmid transposon library mutants with transposon insertions within the ferredoxin ORFs, the fosmid transposon librariers were screened via PCR using combinations of the tranposon-specific FP1 and RP1 primers with one of the ferredoxin cloning primers. A single positive was found, pFOS3G7TnD2 (Figure A4.18), but sequence analysis of the clone revealed that the insertion was not inside of the ORF.

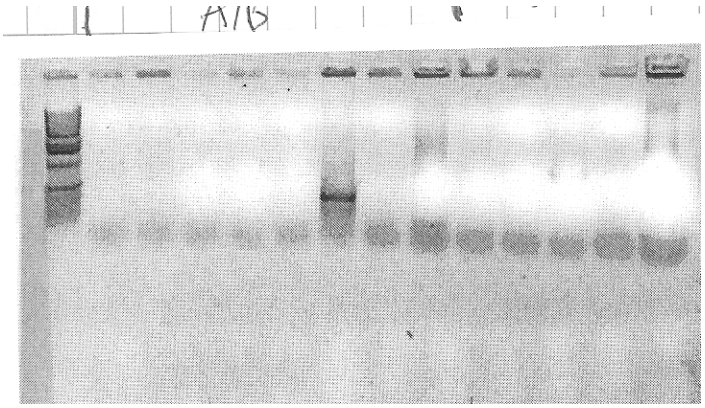

Figure A4.18. PCR screening of the fosmid transposon libraries for mutants with a transposon inside of a ferredoxin-endocing ORF. The single positive result is shown.

Cloning pJ25 into Novosphingomonas aromaticivorans F199

E. coli epi300 pJ25 showed very poor ibuprofen and phenylacetic acid metabolism despite having every *ipf* ORF shown to be necessary for activity in Ibu-2. pJ25 was cloned into the aromatic acid degrader *Novosphingomonas aromaticivorans* F199 in the hopes that it would provide a missing factor of some sort that would allow for the expected pJ25 activity. pJ25 was cloned into J199 by chemical transformation and incubated with or without 500ppm PAA in the presence of 100ppm p-tolylacetic, 1.5mM ferric chloride, 1mM IPTG, and 10ppm chloramphenicol. After a week of incubation, growth of F199 pJ25 was very poor. It would also prove to grow very poorly in the absence of test substrates. This strain would prove difficult to resurrect from the culture collection. This line of inquiry was dropped.

ipfF toxicity assay

Observations of impaired growth in *ipf* clones led to the general hypothesis that *ipfF* has a growth inhibitory effect. Specifically, it was hypothesized that the putative ligase IpfF leads to the accumulation of reactive coenzyme A thioesters which can react

non-specifically with vulnerable intracellular targets. A simple toxicity assay was designed in order to test this hypothesis. *E. coli* JM109 with pGEMt-easy with and without the *ipfF* insert was grown and then exposed to a variety of ibuprofen or 2-(4-tolyl)propionic acid concentrations. The ability of the strains to grow in the presence of the test substrates was quantified by dilution plating. Specifically;

The strains were grown overnight on LBamp. Eight dilutions of the culture were created, the first at 1/1000x, which was sequentially diluted 1/10x. Three 10ul of each dilution was spotted onto seven different plates. The plates were LB with 150ppm ampicillin with 1mM IPTG with;

-nothing

-5, 50, or 500 ppm ibuprofen

-5, 50, or 500 ppm 2-(4-tolyl)acetic acid

All compounds were added to the LB prior to pouring the plates. The fifth quadrants were counted because they had sufficient colony numbers for statistical testing while offering sufficient colony distinction so as to allow clear counting.

The presence of ipfF in the proper orientation was screened for via PCR using ipfF774F / T7 primers (figure A4.19).

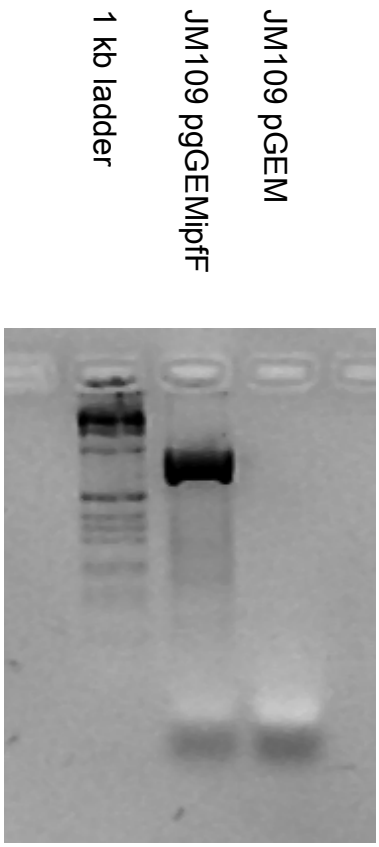

Figure A4.19. PCR amplification of JM109 with the indicated plasmid using the T7F/Ipf774F primers.

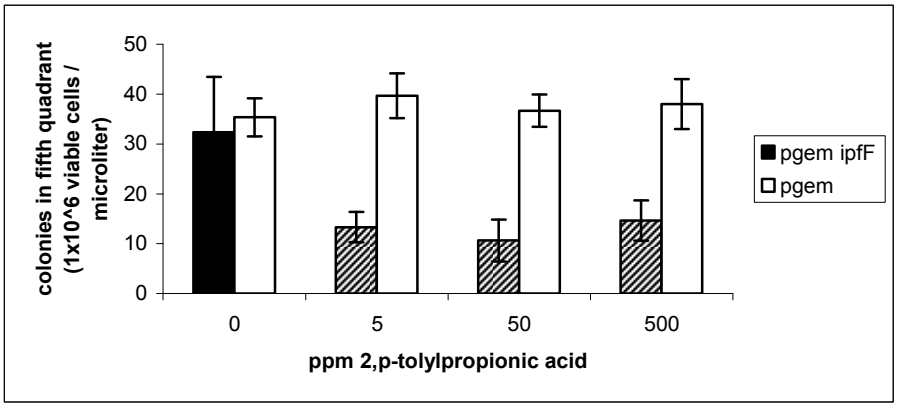

Figure A4.20. Millions of colonies per microliter of overnight JM109 harboring the indicated plasmid incoculated onto LB with the indicated 2-(4-tolyl)propionic acid concentration. Significant inhibition of growth as determined by t-test is indicated by a hashed bar.

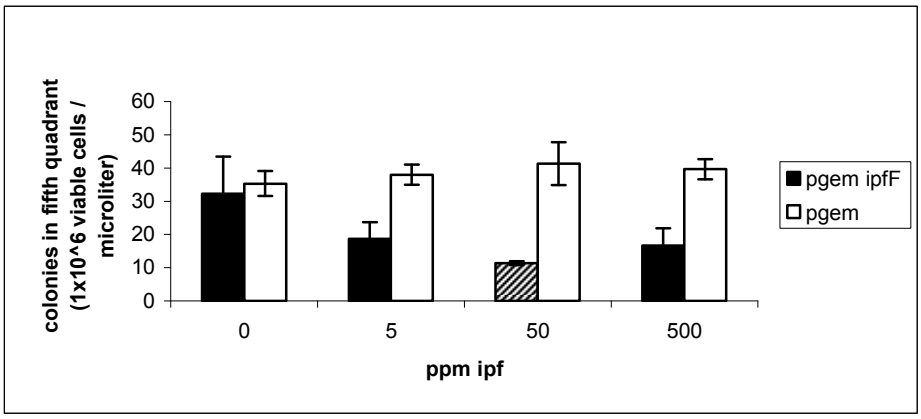

Figure A4.21. Millions of colonies per microliter of overnight JM109 harboring the indicated plasmid incoculated onto LB with the indicated ibuprofen concentration. Significant inhibition of growth as determined by t-test is indicated by a hashed bar.

The results clearly show a reduction in viability of the ipfF-expressing strain when exposed to any 2-(4-tolyl)propionic acid concentration (A4.20) or 50ppm ipf (A4.21). High variability in the pGEM:*ipfF* control (LBamp) spotting likely masked significant differences in the 5 and 500 ipf plates. There was no apparent dose-dependency.

These results seemed to suggest that about half of the ipfF-expressing cells were unable to grow when exposed to the supposed toxin. It seems more likely that either growth is slowed by the stressor or that most cells are unable to grow without a mutation. Neither of these reasonable hypotheses were supported by these results. One explanation that is in line with the results might be that the observed data is simply due to what stage of growth each individual cell was at first exposed to the toxin. For example, a young cell might be unable to survive the initial exposure while a mature cell could.

Regardless, this data was inconsistent with the hypothesis that *ipfF* is generally toxic whether or not substrate is present. However, this assay did not address growth rate, which could still be severely impacted by *ipfF* expression.

Environmental Work

Spiking sewage sludge with ibuprofen and 3-fluorocatechol

The capacity for 1L activated sewage sludge to metabolize ibuprofen to isobutylcatechol was explored by spiking in 500ppm ibuprofen, incubating for six days, then splitting the sample into two; with and without presence of 0.1% 3-fluorocatechol (3FC). The supernatant analyzed via GC/MS. 50 ppm 3FC is sufficient to inhibit meta-cleavage enzymes in pure cultures. An initial assay was performed with 50 ppm 3FC. However, an HPLC analysis of the supernatant failed to detect any 3FC, leading to the hypthesis that 3FC was being sorbed to the extensive solid suspended phase of the sewage sludge or was being otherwise chemically modified. In order to find a concentration of 3FC that would stay in solution for the duration of the assay, increasing amounts were added until approximately 50ppm was detectable in the supernatant via HPLC. Via this approach, it was found that 0.1% was required. At the termination of the 30 minute assay, culture supernatant was recovered by centrifugation, filtered, and subjected to aqueous acetylation and methylation derivitiation.

While several novel peaks were found in the 3FC treated sample, none of them corresponded to any previously hypothesized ibuprofen metabolites, neither isobutylcatechol nor 2,3-dihydroxyibuprofen, nor were they recognizable by comparison to the NIST database. However, many of the novel peaks had the two-loss-of-42-mass-units signature of diphenolic hydroxyl groups. All had masses above that which would be expected for an ibuprofen metabolite. Some representative catecholic ion mass fragment patterns included 350/308/266 (21.4 and 21.75 min.), 380/338/296 (21.7 min.),

366/326/282 (which occurred in four separate GC peaks; 21.8, 21.9, 22.3, and 22.4 min.), and 382/340/298 (20.5 min.). Both samples contained a peak with a fragmentation pattern consistent with the human ibuprofen metabolite carboxyhydrotropic acid (222/191/**163**/132/104).

Spiking bacterial fractionated sludge with ibuprofen

Taken altogether, the sludge 3FC assay results suggested that the Ibu-2 style metabolic pathway was not overwhelmingly employed by bacteria in the sewage sludge sample but that rather some unidentified ring-hyrdoxylation leading to meta-cleavage was occurring. However, if the apparently high capacity for the sludge to sorb or react with 3-fluorcatechol also applies to isobutylcatechol (K_{ow} = 2.997, KOWWIN), then any trace amounts of isobutylcatechol produced would not be expected to be detected. In order to address this hypothesis, a project was designed in which the bacterial fraction would be removed from the sludge in order to decrease the threshold of detection for catechols. A protocol employing acid-washed polyvinylpyrolidone and differential centrifugation was applied to sludge and Cayuga lake water. However, the putative bacterial fractions showed no ibuprofen metabolism and streaks onto LB solid media suggested that very little biomass was recovered from the fractionation protocol, leading to the adjusted experimental design described below

Isolation of additional ibuprofen-growing organisms

Furthermore, twelve additional isolates were streaked from an enrichment culture and subjected to 16S DNA amplification using the 1055F/1492R primer set and

sequencing with 1055F. BLAST analysis of the colonies revealed that many of the isolates were also Sphingomonads (Table A4.3).

Table A4.3. Nearest characterized NCBI nucleotide sequence to the 1055F/1492R amplicon of each of the twelve isolates as determined by BLAST analysis.

species	%identity	range of identity	NCBI accession
Sphingomonas sp.	83	403	X94099
Delftia acidovorans	77	304	AF538930
Sphingomonas sp. KMG425	90	406	X94099
no similarity, degraded sequence	n/a	n/a	n/a
Sphingomonas sp. KMG425	92	56	AB074714
Sphingomonas sp. KMG425	88	403	X94099
Pandoraea sp. G3307	86	403	AF247699
no similarity, degraded sequence	n/a	n/a	n/a
no similarity, degraded sequence	n/a	n/a	n/a
Burkholderia sp. MN101	85	402	AJ555477
Alphaproteobacterium S-A(3)-(A)	87	41	AB074714
no similarity, degraded sequence	n/a	n/a	n/a

Incubation of environmental bacterial fractions with ibuprofen and m-tolylacetic acid

Partly because the biomass recovery from bacterial fractionation of environmental samples had proven too low for rapid metabolite generation, bacterial fractions were incubated with ibuprofen and 3-tolylacetic acid (3TAA) for much longer periods of time and were monitored for accumulation of ring cleavage metabolites.

Three 1L samples were taken from both the Ithaca municipal sewage treatment plant and from Cayuga lake (at the Stewart Park pontoon bridge) and subjected to bacterial fractionation. The fractions, suspended in MSM, were spiked with 500ppm of either R/S-ibuprofen, S-ibuprofen, or 3TAA and incubated for six days total. Disappearance of substrate was monitored via HPLC. At six days, the sludge sample had degraded 100% of the S-ibuprofen and 3TAA while only degrading 21% of the R/S-

ibuprofen. After spiking again with 500ppm of substrate and incubating overnight, each sample supernatant contained a slight acid-labile yellow color consistent with meta-cleavage product. The UV-vis spectrum was characterized against an MSM blank, but the color was too weak to determine a definitive λ_{max} (Figure A4.22).

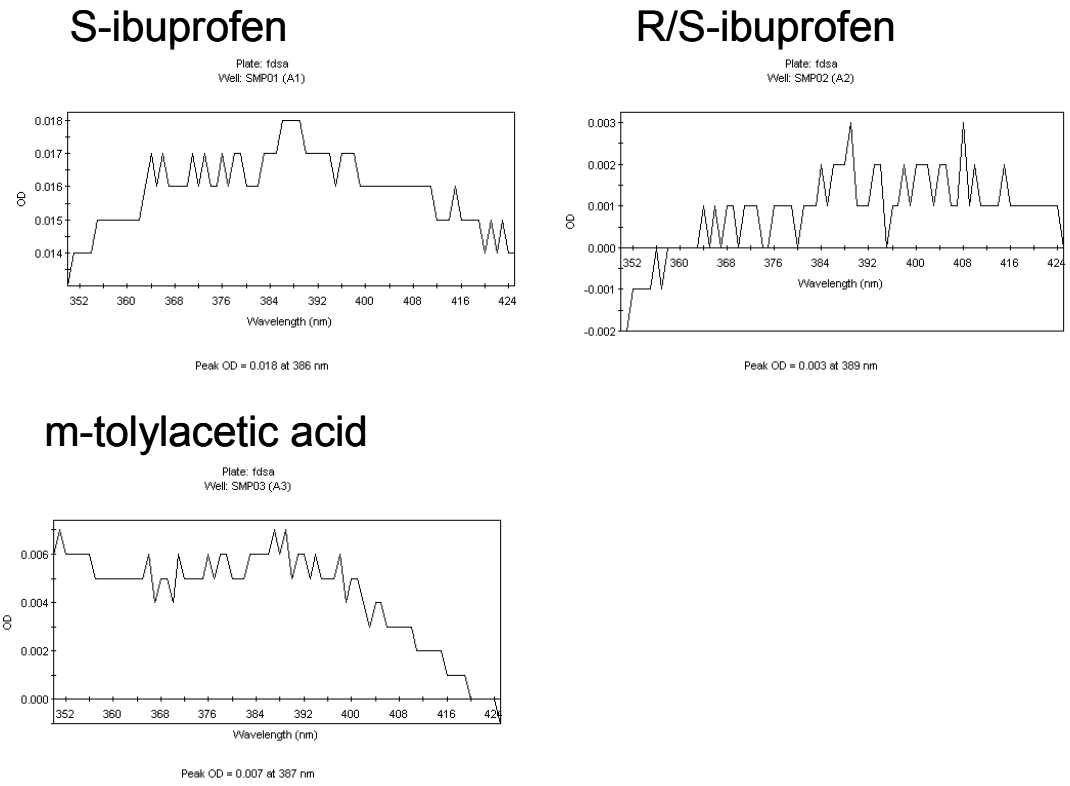

Figure A4.22. UVvis spectra of the supernatants of sludge bacterial fractions exposed to 500ppm of the indicated aromatic acid for six days.

The lake bacterial fraction did not degrade ibuprofen, though a slight amount of 3TAA was degraded. The supernatant of the 3TAA-exposed sample accumulated an acid labile yellow color which was concentrated by back extraction (acidification, ethyl acetate extraction, and re-extraction with a smaller amount of neutral pH water). The λ_{max} of the concentrated extract was 388nm, while the λ_{max} of the meta-cleavage product of 3-methylcatechol, the metabolite generated by Ibu-2 when it grows on mtaa, is 380nm,

suggesting that the predominant mcp was not consistent with Ibu-2 type metabolism. The supernatant absorption spectrum showed a fairly wide hump ranging across the wavelengths commonly associated with many other meta-cleavage products though, so the presence of the mcp of 3-methylcatechol cannot be ruled out (Figure A4.23).

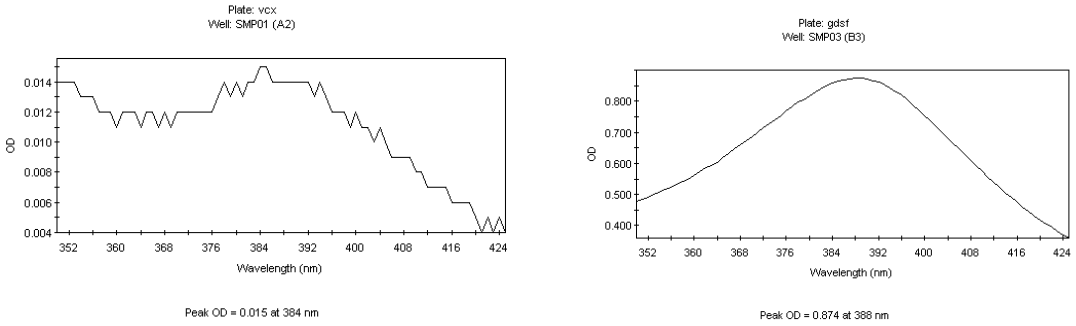

Figure A4.23. UVvis spectra of the supernatants of Cayuga lake bacterial fractions exposed to 500ppm of m-tolylacetic acid for six days. Raw supernatant is shown on the left, while back extracted concentrated supernatant is shown on the right.

The identities of the meta-cleavage products or catechol substrates was not further investigated, though this experiment demonstrated that environmental samples have the capacity to degrade ibuprofen and 3TAA via metacleavage pathways over a fairly short period of acclimation when compared to traditional enrichments, which frequently go through three cycles of acclimation and incubation with the compound of interest.

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APPENDIX 5

IBU-2 DNA SEQUENCES AND ADDITIONAL PUTATIVE PROTEIN CHARACTERIZATION

This appendix contains large DNA sequences that were assembled from individual sequencing reaction results using primarily fosmids 3G7 and 4F6 as template. Three contigs that contain the putative genes described in this dissertation are included. The contigs were assembled using SeqMan (DNA Star, Madison, WI); the alignments for each contig were studied by eye for discrepancies and repaired manually. A brief list of putative ORFs and BLAST gene homologies precedes each of the three contigs listed here. Additional sequences and small contigs are contained in an attached data DVD.

Assembled Sphingomonas Ibu-2 DNA sequences

Contig 1, 11741 bp

11>526 Transposase
840>1718 Catechol 2,3 dioxygenase
2074>3378 *ipfA*
3485>4021 *ipfB*
4030>5229 *ipfD*
5242>5640 *ipfE*
5665>7246 *ipfF*
7342>8481 *ipfG*
9337>8543 Transposase
9800>10809 Catechol 2,3 dioxygenase, incomplete
10818>11150 Ferredoxin
11740>11209 AAA+ ATPase

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        1 AATTCGGTGA ATGCTGCACC AAAGCTTGGC GCTTTTGTCG GGTATTGAGC CGTGGCAGCT
       61 TTTTTCCCAA GTGTTTCAGG CTTCGCAAGG GCCTGGCCCA GGAACCGCTT CGTCTTGGCG
      121 CTGGGGGTCG GCACAGGTAG AAATCGATCG TGTCGCCCCG CTTGTCGACT GCCCGGTACA
      181 GGTAGGTCCA CTTGCCCCGC ACCTTGACGT AGGTTTCATC CAGGCGCCAG CTCGGATCAA
      241 AGCCACGCCG CCAGAACCAG CGCAGCCGCT TCTCCATCTC CGGGGCGTAG CACTGGACCC
      301 AGCGATAGAT CGTCGTATGG TCGACCGAAA TGCCGCGTTC CGCCAGCATT TCCTCAAGGT
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  361 CGCGATAGCT GATCGGATAG CGACAATACC AGCGCACCGC CCACAGGATC ACATCACCCT
  421 GGAAATGGCG CCACTTGAAA TCCGTCATCG TTCCGTCCGT CCAATCTCCG CCAAGCATGC
  481 TCAAGCTTCA CGATTTTTGC AACAGAGCCC GGCCTGGGAT TCATGAATCT CAGCACGCGG
  541 TGCGGTGGGG TAGTGCGTGC CATCGGATGG ACCATACTCA TGATGCGTTC TCCTCAGTAC
  601 CAGAGGCCAA AGGCCCGCCA ACAGGGGCCC GAAACAACGG CCAGCTGCGA CGGGGTACAT
  661 AATCGCACGG AACTCGACAC CTAGGGTTTT TCCCTATCGT AAAAATGTCG AATGTCGATT
  721 ATATTATCGA GATTATGACG AGACACACGT TGCTGAGCCT TGATTCGGTC CATGCTGAAG
  781 TGTGGCGAAA TCTTCTGTAA TGACCAAGTG AAAAATACAA TGAATGCGAG AACTTCAAAA
  841 TGAGTATCAA AAGCTTGGGT TACATGGGAT TCGCGGTCAA GGATGTGCCC GCCTGGCGTT
  901 CTTTCCTGGC CCAGAAATTG GGCTTGATGG AAGCAGGCGT CACCGATGAC GGCGCCTTGT
  961 TTCGCATCGA TTCGCGCGCC TGGCGGATTG CCGTCCAGCA GGGCGAGGCA GACGATTTGG
 1021 CCTACGCTGG CTACGAGGTG GCCGATGCAG CGGGTCTGAC GCAGATGACA GAAAAACTGC
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 1141 TGGGCTTGAT TTCGTTTGCC GATCCGTTTG GTCTGCCACT GGAAATTTAT TATGGCGCCA
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 1261 AGCAGGGGCT GGGTCATTTT GTGCGCTGCG TGCCGGATTC GGACAAGGCG CTGGCGTTTT
 1321 ATACCCAGGT GCTCGGCTTC CAGTTGTCGG ACGTCATCGA CATGAAAATG GGACCGGACG
 1381 TGACGATTCC GGCGTATTTT CTGCACTGTA ACGAACGCCA CCACACCCTC GCGATCGCAG
 1441 CTTTTCCGCT GCCCAAGCGC ATTCACCACT TCATGCTCGA AGTCTCGTCG CTCAATGACG
 1501 TCGGTTTTGC GTTTGACCGG GTTGACGCTG ACGGCTTGAT CACCTCCACG CTGGGGTGCC
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 1621 ACGGCTGGGG CGCCCGCACC GTTGACCGCT CCTGGGTGGT GGCACGGCAC GATAGCCCGA
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 1801 TTGCGCGAGG CGGGCCTGAA CTTCGAGCTG GTACAAGTCG ATCTGACATG ATTGAAGGTC
 1861 GCACCGAAGA GCAAAAACGC GCGGTGATCG AAAAGGTCAC GCAGGCGCTG GTTGACGCTG
 1921 TCGGCGCGCC CAAGGAAAAC GTCAGAGTCT GGATCCAGGA CGTGCCCAAG GAGAACTGGG
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 3181 GACAGCGTGA TTTCGGAGCG CCAGCAAATC GCCTTGCGCG ACCGTGCCGA CTGGCTGGAC
 3241 GTGTCGCGCG GTCTGAACCG AGAGCATGTC GATGAGATGG GCGTGGTGGT GGGGCACGTG
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 3361 TCGCCGTCCC CTGTTTGAGC GTATTGATCA CCACCTCATC CGCCCCCCTT CGGGGGCGGC
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 3841 ACTGTCGAAC TTTGTGCTTT ACGAATTGCG GCGCTCATCG ACAGGTCAGA TAGAAGTTTG
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 4081 CTGGGTTTCA GTGCGATGTC ACGCCAGCCC GTCGGCACGA TCCGCGAGTT GGCGGCCACT
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Contig2, 8229 bp

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1231>2028 4-oxalocrotonate decarboxylase
2032>2244 4-oxalocrotonate isomerase
2226>2558 Plant-like ferredoxin (*ipfI*)
3636>2593 Transposase
3798>5052 Ferredoxin reductase (*ipfH*)
5533>5102 *xylL*-like dehydrogenase (*ipfL*)
5561>6502 Transposase
7044>8229 Transposase

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      7381 CACCGCATGA AGCGGGTGGA TCGCGCGCAA TTGACGGAAT GGATTGCTGG CGAGCTGTGC
      7441 CCGGGCGGCC AGTCGATCAA TGCCATGCTT GAGCATGTTT TCCTGTGGTG CCGGGACCGG
      7501 CGTATTTATG GGCCGTCGCG CAAGGAGCTT GAACGTGTCG TTCGCTCACA ACGGCAAGAT
      7561 TATCTGGACA CCTGGCTGAT CGGAGCCAGT GATCGGCTTT CGTCAGATGC GGTGGCGTTA
      7621 TTGGAAGCCT CGCTTGCCGA TCCGGACAGC TCGACCGGAT TCAACAGGAT GAAGGGTGAC
      7681 GCCGGACAGG CAACGCTCGA CAACATTCTC GACGTGACCG AGAAACTCGC CTTTATCCAG
      7741 AGACTTGATC TTCCCCATGA TCTCCTGACG GCTACGGGCA AGCCATGGGT CGATCAGATT
      7801 GTTCGCCGCG TTGCCGGTGA AAAGGCCTCG GAGATGCGCC GGCATGCGCC GGCGCGACAG
      7861 CTCGGCCTTT ATGCGATTTA TCTAATGTCG CGGGAGGCGC AACTCACTGA CGCGATGATC
      7921 GACCTGCTGA TCGAAACCGT TCACAAGATC GGAACGCGCT CGAAACGCAA GGTGGTGGGC
      7981 GATATCGCGA AAGACATCGA GCGGGTCTAT GGAAAGGAGC GCCTGCTGGT CGAGATCGCC
      8041 AGCGCCTCGA TCAATGAACC ATCGGGGCGC ATCTGCGATG TCATTTTCCC GATCGCCGGT
      8101 AAGGCCAAGC TGGCGGCGAT CGTCAAGGAG AGCCATGCGA AGGGCGCTCT GGACCKGCGC
      8161 ATCTACAAGG TGATGTGTCA ACGGCGGAGC AAAAGTCGGC CATTCGGCGG CGTAAAACCA
      8221 GGCCATCGT
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Contig3, 1954 bp

1047>247 Cinnamoyl CoA-hydratase
1954>1104 Acyl-CoA transferase / racemase

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         1 GGGCAGTGAG CGNAACGCAA TTAATGTGAG TTAGNTCACT CATTAGGCAC CCCAGGCTTT
        61 ACACTTTATG CTTCNGGNTN GTATGTTGTG TGGAATTGTG AGCGGATAAS AATTCACCGG
       121 AAAGCTATGA CCATGATTAC GCCAAGCTAT TTAGGTGAGA CTATAGAATA CTCAAGCTTG
       181 CATGCCTGCA GGTCGACTCT AGAGGATCCC ACGATATTCA TTCAATATTC CTATTTCCTA
       241 TTGTCGTATA GTTTTCTAGC GCCGGTTTGA ATTTTCGATT AGAGAACCCC TGAAGTGCGA
       301 CGTCAAGCCA TTTCTGACGC GATGTATACG AAAGTTCAAA GAATTTTGCT GATTCCCATT
       361 CAACTGCTTC GTCGAAATCC ATTTCGACGG ACCGCTCGAA TACACGCTTG ATCGCGACAG
       421 CCGCGTGCCT GTCTTTTGCT GCCAACTCGG TGCTCAGCCT GTCAATCTCG CCGCTCAGAT
       481 CTGCAACCGG AACCACATGA TTGACCAATC CGATCTCCGC CGCCTTGTGA CCGTCGAACA
       541 CCTGCCCCGT CAGGAGGTAA TAGAGCGCCA CTTTGCGTGG CAAGTGCCGG GCGACCATCC
       601 AGGTCAGACC TGCACCGGGG ATGACGGCGA AGTTGATTTC CGAAAGGCTG AAACGCGCGT
       661 TCGAGGCAGC GACCGACAAA TCGCAGAGCC CCGCGACCAA AATGCCTCCG CCCACGCACC
       721 AGCCCTGAAC CGCCGAGATC GTGATGCCGT TAAAGTGGCG AAGACGCTTC ATCCACGACA
       781 GCGCCAGATC CATCGTTCGC ATGAACATGT CGGAGTCCCC GAAATTCCCA AGCAGGAACT
       841 CCTCAAGATC CATGCCTGCG CAGAAGTTGT CACCTCTCCC GACAATAACG ATAAGCCGTA
       901 CATCGCCCCG GCGCTCCACC TCGTCCAGCG CGGAGTGCAT GTCGGCATGW ACTTGCGGAG
       961 ACATCGCGTT GCGCTTTTCG GGGCGATTGA ACKCGATCGT TGCTCGCGCT CMATCGAAAC
      1021 ACAGGTCGAC GGTCGGAAAA GTTACGGCGT TCATGTCGTC TCTCCCGTTG CGGCCCGGCC
      1081 GACCCCGGTC TCTTCGCGTG CCCGCGCTTC ATCCGGTTCA CCGAAATAGC GATGATTATG
      1141 CTCACCCAGA GCGGGTGAAG ATGCCGTGGA GTCGGGCGTG TATCCGCTGA TCTGTATCGG
      1201 GAGTTCGAAT ATCACGTCTG ACGACGCTAT CCCGGCACAG CCCGCTTCCG CCTTTCCGTT
      1261 CGCCATGGCT TCGGCTACGC TAAGCACGGG AGCGAAACAA CAGTCGGCCT TCACCAGCAG
      1321 GTTGCACCAG TGATCAAGTG GGTGCGACCC AAAGATTCCT GCAAGCTCGG ACTTCAGCCA
      1381 GGCTTGGCGA GTCTCGTCGA ACTGGAAGGG CTGCAAGTCG GGCCTCTGCA GCGCCGCACA
      1441 CAGCCTCGCC CAGAACTTGG GTTCACCGGC CCCGACGGTC AGATGGCGAT AGTCAGATGT
      1501 CTGATATATC TGGTAGCAGG CAAAGCTCCC GTCGAGAAGC GAAGGCCCGC CGGCCGACAT
      1561 CGTTAGCGGC CCGATGTTCA GCGGCTTGCA GCATTCGGTC ATGGACACGC CGTAGAACCG
      1621 TCCCCGGCCC GTCCGTTGCC GCTCGACGAT GCCGGTCAGC ACCGCGATCA CGGCGTGAAG
      1681 AGATCCACCC ATGACGTCCG CGATCTGGAA ACGCGGCAAA ACCGGCCCTC CGGTATCATC
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1741 ACGCAACTGA TCCAGAAGCC CCGCGGTATG CAAGGTAATT CAGATCGTGG CCCGCGAGAT
1801 CGCTGAAGGG AACCAGACTG ACCATAACCG GTTAATCGAG ACATAGACGA NCGCGGGCTT
1861 GATANCCNAA AAGANTCGCG TAATCNATTC CAANGCTTCG CTGCTAACGC CCGGCCTAAA
1921 GCTTTTCCAN NAAAAAAATC CGCTTCGCGC AACA
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Phylogenetic analyses of putative gene products of ipfA and ipfB

The figures in this section represent additional results of phylogetic analyses performed on IpfA and IpfB as described in Chapter 3.

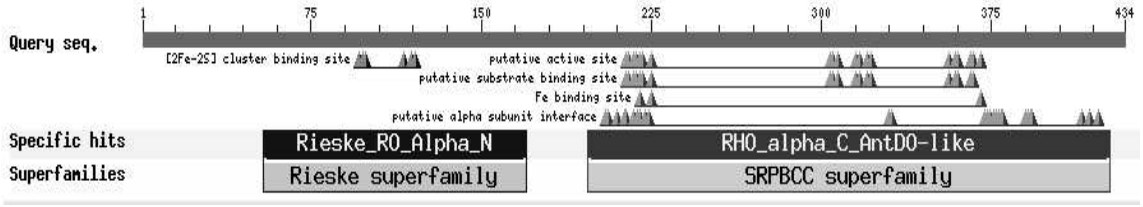

Figure A5.1. Conserved domain architecture of the predicted protein encoded by *ipfA* obtained via CDD analysis.

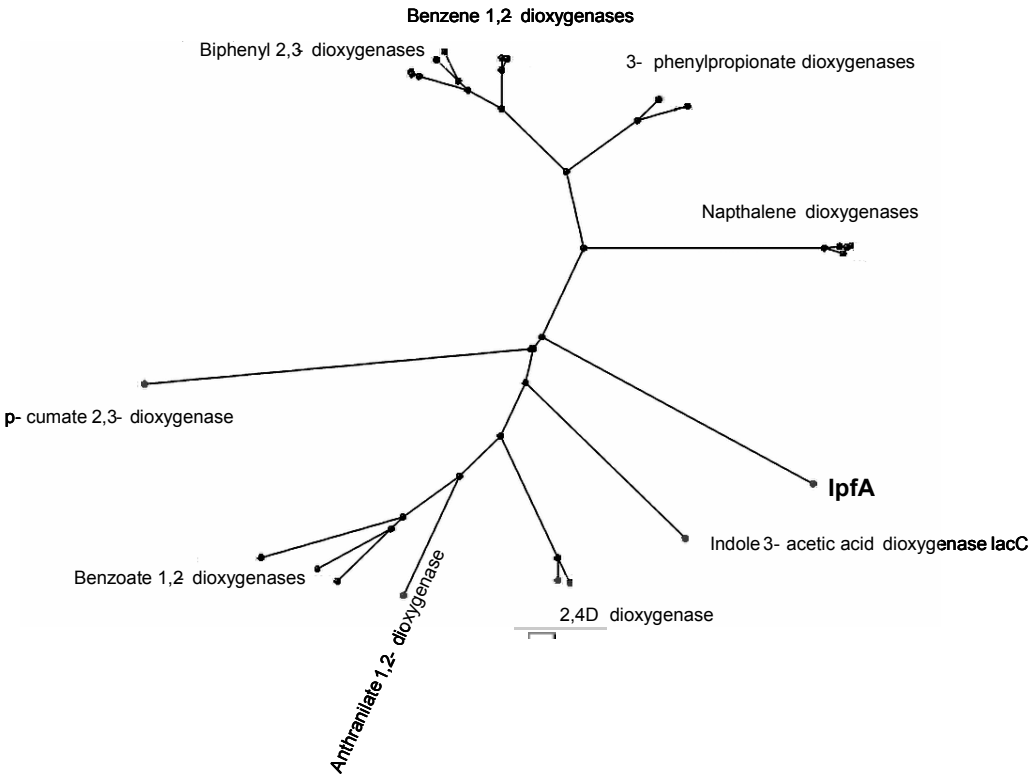

Figure A5.2. Phylogenetic tree of the predicted amino acid sequence of *ipfA* and characterized dioxygase large subunits found in the Swiss-prot database and listed in Table 3.4.

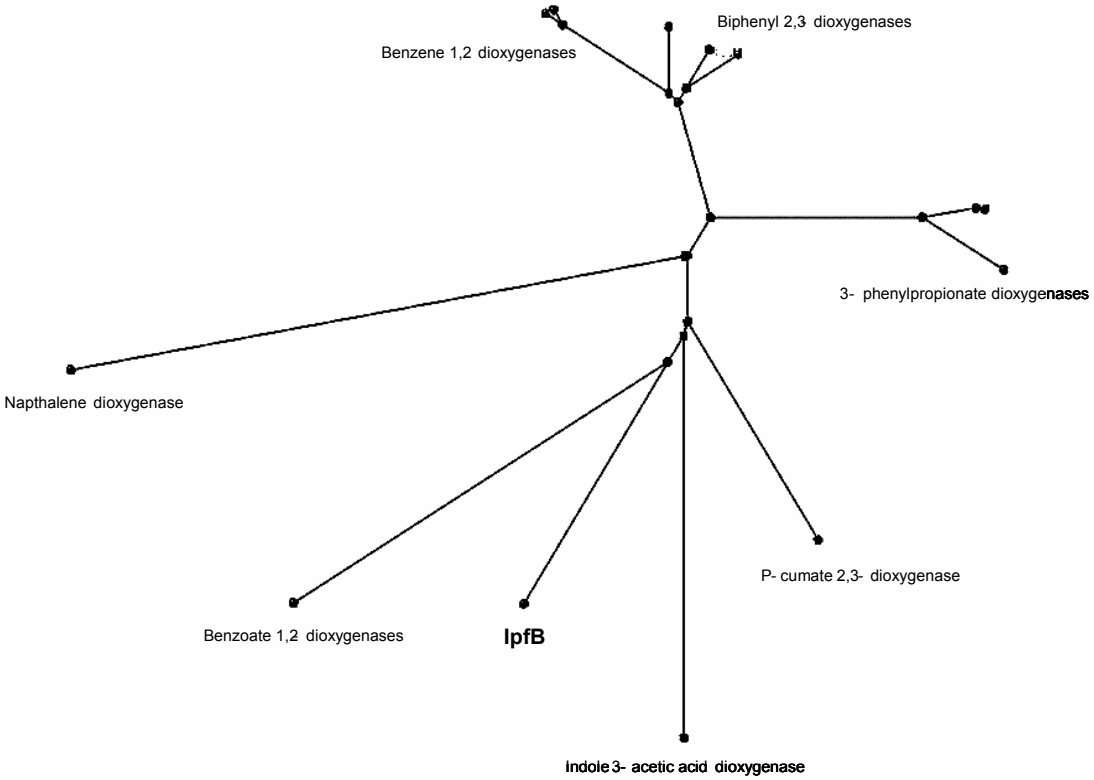

Figure A5.3. Phylogenetic tree of the predict amino acid sequence of *ipfB* and characterized dioxygase small subunits found in the Swiss-prot database and listed in Table 3.5.