ADAPTATION OF PSEUDOMONAS PUTIDA F1 TO GROWTH ON STYRENE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2011

ADAPTATION OF PSEUDOMONAS PUTIDA F1 TO GROWTH ON STYRENE

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Cornell University 2011

Pseudomonas putida F1 is unable to grow on styrene, a common industrial pollutant, despite degrading it through the toluene degradation (*tod*) pathway. This dissertation investigates the biochemical and genetic aspects of styrene degradation by *P. putida* F1 and identifies substrate-level catechol-2,3-dioxygenase (C23O) inactivation as the factor which prevents growth on styrene. Novel adaptations to growth on styrene were investigated and found to mitigate C23O inactivation through management of 3-vinylcatechol production and consumption.

Previous studies of *P. putida* F1 identified TodF, a hydrolase responsible for the degradation of styrene *meta*-fission product (MFP), as putatively responsible for F1's inability to grow on styrene. Through kinetic and growth analyses, we demonstrated that TodF is capable of styrene MFP degradation and thus not responsible for prohibiting growth on styrene. Preliminary genetic analysis of styrene adapted mutants strengthened this conclusion.

It was found that cultures of F1 exposed to styrene accumulated 3-vinylcatechol in the media, suggestive of inhibited C23O activity. Specifically, micromolar concentrations of 3-vinylcatechol were found to inactivate TodE during catalysis. Analysis of cells growing on styrene suggested that inactivation of TodE and the subsequent accumulation of 3-vinylcatechol resulted in toxicity and cell death. Over-expression of TodE or an inactivation resistant C23O on a plasmid was able to prevent catechol accumulation and confer the ability to grow on styrene.

We characterized a spontaneous F1 mutant, designated SF1, which was capable of growth on styrene and did not accumulate 3-vinylcatechol. Resting cell

assays demonstrated that the activity of toluene dioxygenase (TDO), the multi-component enzyme responsible for the production of 3-vinylcatechol from styrene, was reduced in SF1. DNA sequence analysis of the *tod* operon revealed a single base pair mutation in *todA* (C479T), a gene encoding the reductase component of TDO. Replacement of the wild-type *todA* allele in F1 with $todA_{C479T}$ from SF1 reduced TDO activity, prevented 3-vinylcatechol accumulation, and conferred the ability to grow on styrene. Collectively, our data reveals a unique adaptation where slowing down the rate of vinylcatechol production via decreased TDO activity leads to reduced C23O inactivation and permits growth on styrene.

BIOGRAPHICAL SKETCH

Kevin George was born and raised in rural southern New Jersey, spending most of his time in the small town of Clarksboro. From a young age, Kevin was fascinated with science and the natural world. His father recounts observing him as one-year old, staring contently at ants on the back porch for hours at a time. Kevin's love of science was further nurtured by his older brother Michael, who exposed him to astronomy – a source of endless wonderment – and introduced him to the awe-inspiring theory of evolution at an early age. Kevin attended high school at Kingsway Regional in Swedesboro, NJ, and had the privilege of attending the Governor's School in the Sciences at Drew University the summer of his junior year. For 2 months, Kevin took courses in diverse subjects such as Ecology, Evolution, and Biochemistry and came away with a profound appreciation for both the problems facing the natural world and the ability of experimental science to address them.

Kevin attended college at School of Environmental and Biological Sciences at Rutgers University in New Brunswick, New Jersey, receiving a B.S. in Biochemistry in 2006. While at Rutgers, Kevin had his first experience with research, working in the lab of Dr. Max Häggblom. Working with Dr. Häggblom, Kevin was first exposed to the concept of bioremediation and the use of microorganisms to degrade pollution. The science of bioremediation allowed Kevin to combine his passion for environmental issues with his love of experimental research. After 3 fruitful years performing biodegradation research at Rutgers, Kevin decided to pursue his Ph.D. at Cornell University in the laboratory of Dr. Anthony Hay. During his Ph.D. research, Kevin furthered his knowledge of biodegradation, working with an extraordinary microorganism capability of degrading a wide range pollutants. In the future, Kevin hopes to apply the vast genetic potential of microorganisms to a variety of environmental problems, from pollution to the production of clean energy.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Anthony Hay, for his support, guidance, and nearly continuous stream of experimental advice. Thanks to my committee members Dr. Eugene Madsen and Dr. David Wilson for their contributions and insight. I thank the staff and faculty of both the Field of Environmental Toxicology and Department of Microbiology who have been friendly and accommodating throughout my time at Cornell. I would especially like to thank Cindy Uhrovcik, whose commitment to students is unmatched. Thank you Cindy for your consistent support and assistance, I do not know what I would have done without you.

I am sincerely grateful to the members of Hay lab, past and present, (Bryan Campbell, Andreas Toba, Giomar Rivera-Cancel, Abigail Porter, Karl Rueggeberg, Mitch Thompson, Amy Risen, Daniela Bocioaga, Gabriela Hidalgo, Jeffrey Peacock, Mary Thompson, Robert Murdoch) for their companionship and extraordinary ability to make me laugh. Hay lab has been an amazing place to work, and I owe much of my positive experience to the good-natured atmosphere you have created. Thanks to Mary Thompson for her experimental assistance, humor, and enthusiasm. Thank you to my remarkable friends at Cornell. To Amanda Solliday, thank you for believing in me and encouraging me to follow my aspirations. Thanks to Rajni Singh for spending way too much time at the Dairy Bar with me and helping me through tough times. Most of all, I want to thank my family for their love and unconditional support. Dad, your encouragement and love has been a consistent source of strength. Thank you for everything.

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BACTERIAL STRATEGIES FOR GROWTH ON AROMATIC COMPOUNDS

ABSTRACT

Although the biodegradation of aromatic compounds has been studied for over 40 years, there is still much to learn about the strategies bacteria employ for growth on novel substrates. Elucidation of these strategies is crucial for predicting the environmental fate of aromatic pollutants and will provide a framework for the development of engineered bacteria and degradation pathways. In this chapter, we provide an overview of studies which have advanced our knowledge of bacterial adaptation to aromatic compounds. We have divided these strategies into three broad categories: 1) recruitment of catabolic genes, 2) expression of "repair" or detoxification proteins, and 3) direct alteration of enzymatic properties. Specific examples from the literature are discussed, with an eye towards the molecular mechanisms which underlie each strategy.

I. INTRODUCTION

Microorganisms possess the extraordinary capacity to degrade a multitude of pollutants, many of which have only recently been introduced to the environment due to human activities. Of these pollutants, aromatic compounds are particularly problematic due to their resistance to degradation and deleterious effects on human and environmental health (ATSDR, 1996, Millennium Assessment, 2005). Given these concerns, it is critical to understand the strategies microorganisms employ to adapt to growth on aromatic substrates.

The recent oil spill in the Gulf of Mexico is a topical reminder of the indispensible role microorganisms play in the degradation of petroleum hydrocarbons and the importance of microbial adaptation. Aromatic compounds comprise on

average 15 % of crude oils and as much as 70 % of gasoline by volume (Hyne, 2001; Tessoro 2003). A recent study of indigenous oil-degrading bacteria in oil-impacted areas of the Gulf of Mexico found that hydrocarbon biodegradation rates were faster than expected and revealed significant potential for intrinsic bioremediation of the oil (Hazen *et al.*, 2010) . Within the plume, the population of oil-degrading bacteria was enriched as were genes associated with the degradation of key aromatic oil constituents such as isopropylbenzene and naphthalene.

The bacterial capacity to metabolize a wide range of diverse aromatic substrates is likely related to the fact that that the common unit of aromatic compounds – the benzene ring – is among the most ubiquitous chemical structures in nature (Harwood and Parales, 1996). This abundance has contributed to the selective pressure that has driven the evolution of a relatively conserved, yet genetically flexible, mechanism for degradation that relies on the addition of either one or two atoms of molecular oxygen to the ring (Harayama *et al.*, 1992). Encoded by a significant amount of genetic diversity, the mixed function oxygenases that perform these reactions have been the subject of both basic and applied research. Much of this research has focused on their potential as catalysts for specialty chemical production (Cirino and Arnold, 2002; Gibson and Parales, 2000) and bioremediation (Gibson and Parales, 2000; Pieper and Reineke, 2000; Urgun-Demirtas et al., 2006).

Despite the vast metabolic potential of bacteria, aromatic biodegradation is not always effective, particularly *in situ*. There are numerous potential reasons for this, ranging from unfavorable growth conditions to limited pollutant bioavailability (van der Meer *et al.*, 1992). In the case of structurally complex xenobiotics such as halogenated pesticides, a more obvious factor is the absence of preexisting bacterial enzymes capable of substrate degradation. Given that the chemical structure of certain xenobiotics may be quite different from the substrate specificity of existing enzymes,

it is not surprising that mutations which expand substrate range or increase degradation efficiency may be required before a bacterium is capable of degrading a compound, let alone using it for growth. In some instances, alternative catabolic enzymes must be "recruited" either from within a bacterium's genome or horizontally from other organisms. Aromatics which are toxic or produce toxic metabolites present additional challenges to bacteria and require their own unique adaptive responses and strategies. The need for these various mutations can often result in extended periods of adaptation prior to pollutant degradation (van der Meer, 2006; van der Meer *et al.*, 1987; van der Meer *et al.*, 1998). Understanding the mechanisms which underlie bacterial adaptation to novel aromatic substrates is likely to yield improved predictions of biodegradation potential and provide a framework for directed pathway evolution (Diaz, 2004).

In this review, we describe some molecular mechanisms and bacterial strategies for growth substrate expansion and improved biodegradation of aromatic substrates. Before providing specific examples, we first review the conserved mechanisms of aerobic aromatic degradation. A brief overview of regulation in aromatic degradation pathways follows, with a concise discussion of genetic organization. The bulk of this review then focuses on genetic adaptations and strategies which allow bacteria to metabolize aromatic compounds. Broadly speaking, we discuss adaptations which fall into three main categories, resulting in either catabolic gene recruitment, expression of specialized "repair" proteins, or direct alteration of enzymatic properties. Though these strategies are discussed separately, it is important to note that they can and likely do occur simultaneously, working in concert to increase degradation capacity, decrease toxicity, and thereby expand growth substrate range.

II. OVERVIEW OF AEROBIC AROMATIC DEGRADATION

Although both aerobic and anaerobic biodegradation contribute to the elimination of aromatic pollutants from the environment, the aerobic mechanism is much more prevalent in the biosphere (Cao *et al.*, 2009) and will be the focus of this review. The aerobic degradation of both mono- and polycyclic aromatic hydrocarbons normally proceeds via two major steps, designated as the upper (or peripheral) and lower (or ring-cleavage) pathways (Diaz, 2004) (**Figure 1.1)**. In the upper pathway, the critical step is the destabilization of the ring through mono- or dioxygenation, typically resulting in the addition of two hydroxyl groups (Mason and Cammack, 1992). The remainder of the upper pathway focuses on preparing the ring for cleavage, typically using dehydrogenation to form a catecholic intermediate. Following the formation of catechol or a closely related monocyclic compound such as gentisate, the lower pathway begins when the hydroxylated aromatic ring is cleaved by a second dioxygenase (Harayama et al., 1992; Harayama and Rekik, 1989; Vaillancourt et al., 2006). Once the ring is cleaved, further transformations usually funnel ring-fission products into the TCA cycle for energy production. Additional detail regarding the upper and lower pathways is provided below.

A. THE UPPER PATHWAY

Due to the stability of both the aromatic ring and of molecular oxygen (O_2), the formation of a reactive oxygen intermediate is a prerequisite for ring oxidation. Ring-hydroxylating oxygenases are responsible for catalyzing the incorporation of oxygen atoms into the aromatic ring. Many of the best described oxygenases are Rieske, nonheme iron oxygenases comprised of multi-component complexes, usually consisting of electron transport proteins which transfer electrons from NAD(P)H to a terminal, iron-sulfur oxygenase (Butler and Mason, 1997; Gibson and Parales, 2000)

(**Figure 1.2)**. The electron transport component often consists of an iron-sulfur ferredoxin along with a separate reductase or a combined ferredoxin-NADH-reductase (Gibson and Parales, 2000). The terminal oxygenase is typically a heteromultimer, composed of a large (α) and small (β) subunit although the arrangement of subunits varies (Furusawa *et al.*, 2004). The α subunit contains a Rieske-type [2Fe-2S] cluster which is reduced by electron-transfer proteins and functions as an oxygen activation center (Butler and Mason, 1997). This subunit is also responsible for substrate binding and recognition (Furusawa et al., 2004; Gibson and Parales, 2000). Following the incorporation of oxygen, dehydrogenation usually takes place resulting in catechol or related structures such as gentisate, hydroquinone, or salicylate (Gibson & Subramanian, 1984).

B. THE LOWER PATHWAY

Following the creation of a catecholic intermediate, ring fission occurs through *ortho*- or *meta*- cleavage. During *ortho*- cleavage, dioxygenation takes place at the 1,2- position of the catechol (within the hydroxyl groups), while during *meta*-cleavage fission takes place outside the hydroxyls and usually occurs at the 2,3- position (Harayama and Rekik, 1989) (**Figure 1.3**). Distal ring fission in the 1,6- position of the catechol has also been reported (Horvath, 1970; Koh *et al.*, 1997). *Ortho*- and *meta*- cleavage are catalyzed by intradiol and extradiol dioxygenases, which use Fe (III) and Fe (II) at the active site to catalyze ring cleavage, respectively (Harayama *et al.*, 1992). Although these differences between the dioxygenases seem trivial, the enzymes have vastly different structures and dissimilar catalytic mechanisms (Vaillancourt *et al.*, 2006). In general, extradiol dioxygenases (catechol-2,3-dioxygenases, C23Os) appear to be more versatile and occur in far more catabolic and biosynthetic pathways (Vaillancourt *et al.*, 2006).

Most products generated by *meta*-cleavage (*meta*-fission products, MFPs) take the form of a common structure, 2-hydroxy-6-oxohexa-2,4-dienoate (HODA), with different substituents on the C6 carbon depending on the substrate (Khajamohiddin *et al.*, 2008). Further degradation of MFPs proceeds via either a hydrolytic or an NAD-dependent dehydrogenase/4-oxalocrotonate pathway(Sala-Trepat *et al.*, 1972) (**Figure 1.4)**. These branches, and the specific reactions they catalyze, are key determinants of substrate range in aromatic degradation pathways: each branch has a limited substrate range that is constrained by the nature and position of the substituent(s). Para- or non-substituted catechols form aldehyde-group containing MFPs and are acted upon by the dehydrogenase pathway, while cleavage of 3-substituted catechols generates a ketone which proceeds through the hydrolytic branch. Since most degradation pathways do not contain both branches, the nature of the lower pathway becomes a key determinant of substrate range. Within the hydrolytic branch, MFP hydrolases possess strict substrate specificities and appear to play crucial roles in determining substrate preference in bacteria (Khajamohiddin *et al.*, 2008).

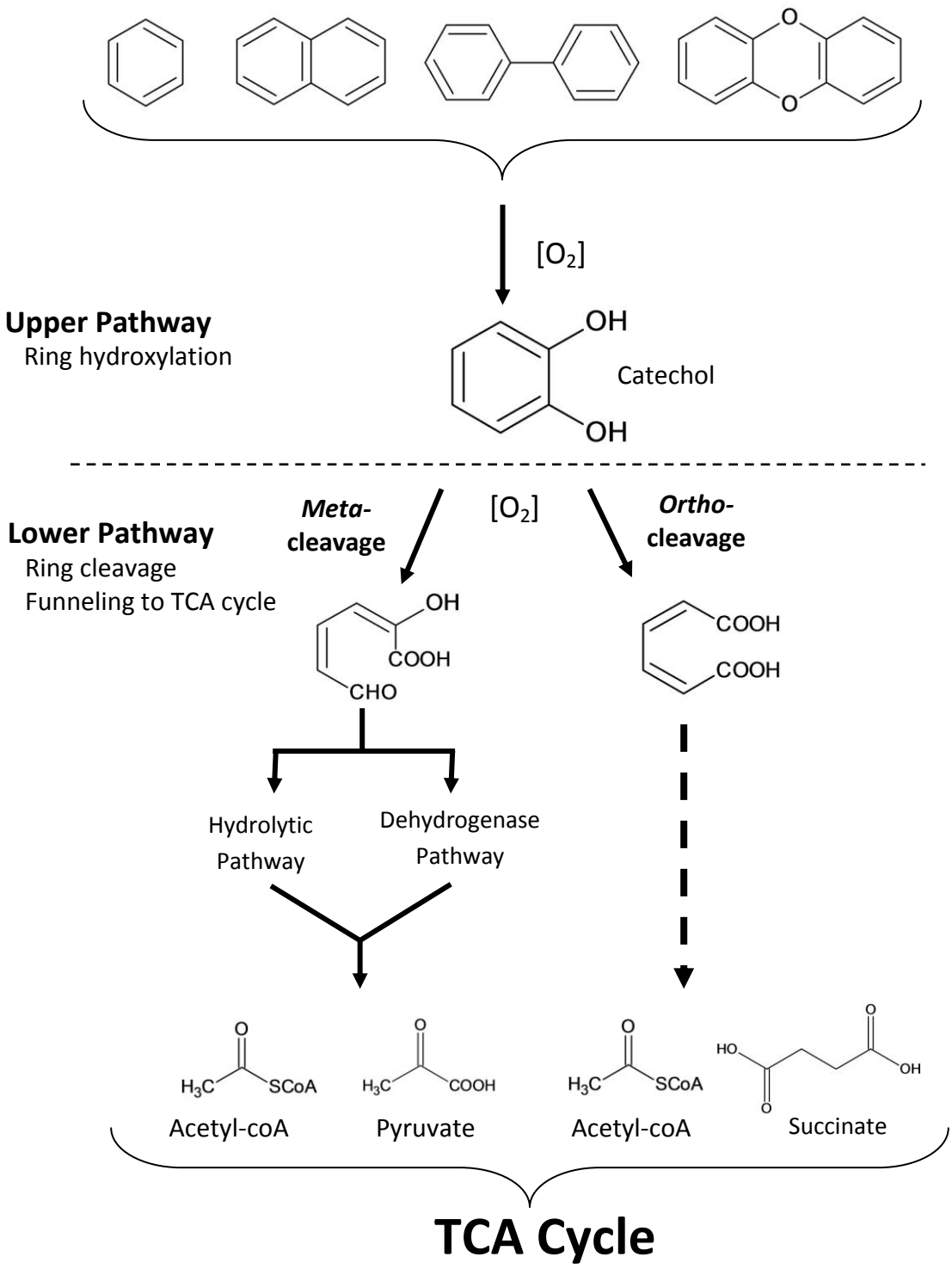


Figure 1.1. General schematic of aerobic aromatic degradation.

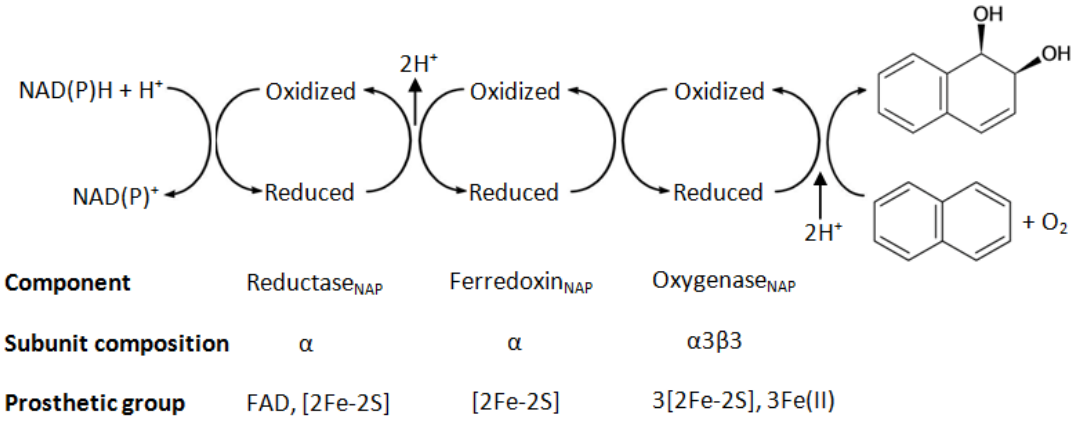


Figure 1.2. Organization of the components of the naphthalene dioxygenase system from *Pseudomonas* sp. strain NCIB 9816-4, a well-characterized ring-hydroxylating dioxygenase. The flow of electrons is illustrated in the top panel, while subunit composition and prosthetic groups are labeled below. Adapted from Gibson & Parales (2000).

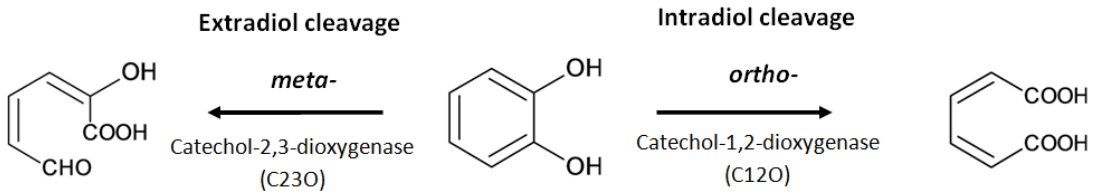


Figure 1.3. Extradiol (*meta*-) vs. Intradiol (*ortho*-) cleavage. Extradiol cleavage takes place at the 2,3- position on the catechol (outside the hydroxyls) while intradiol cleavage takes place at the 1,2- position (inside the hydroxyls). The reactions are catalyzed by catechol-2,3-dioxygenases (C23Os) and catechol-1,2-dioxygenases (C12Os), respectively.

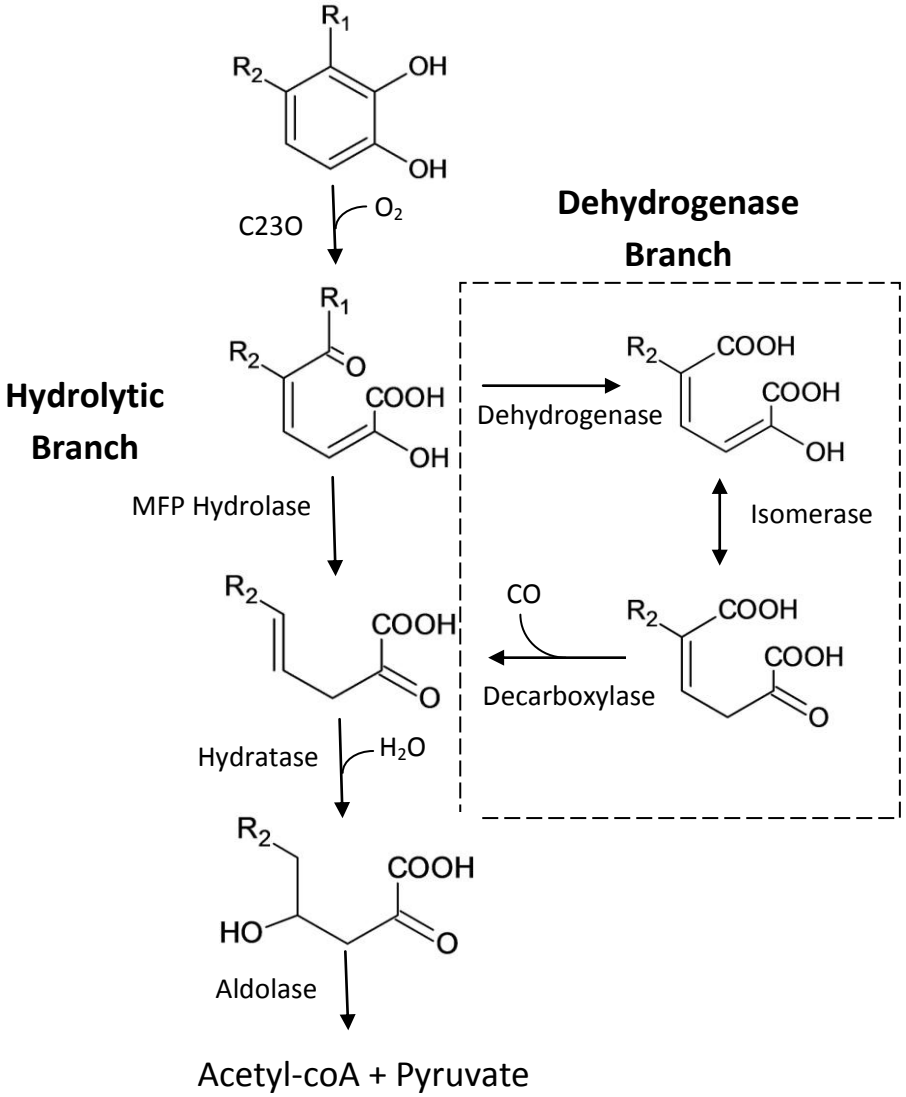


Figure 1.4. ***Meta*-fission lower pathway for catechol and substituted catechols**. The hydrolytic and dehydrogenase branches are denoted. Enzymes typically responsible for catalysis are labeled. Adapted from Khajomohiddin *et al.* (2008).

C. C23O INACTIVATION AND THE PRODUCTION OF TOXIC METABOLITES

Many intermediates produced during aromatic metabolism are capable of causing cellular toxicity (Chavez et al., 2006; Gerischer and Ornston, 1995; Park et al., 2004; Perez-Pantoja et al., 2003; Pumphrey and Madsen, 2007) and require specific bacterial adaptations for degradation. Catecholic compounds, perhaps the most common intermediates in aromatic degradation, are particularly problematic. During the course of catalysis, C23Os may become inactivated by their catecholic substrates in a process known as mechanism-based or suicide inhibition (Bartels et al., 1984; Klecka and Gibson, 1981) which involves oxidation of active site Fe (II) to Fe (III) (Cerdan *et al.*, 1994; Vaillancourt *et al.*, 2002) (**Figure 1.5**). This sort of C23O inactivation has been primarily observed with chlorocatechols, but some alkyl catechols can exert a similar effect (Vaillancourt *et al.*, 2006). C23O inactivation and the subsequent accumulation of catechol has been shown to limit the substrate range of a variety of pathways (see Section III. B. 1).

In addition to suicide inhibition, catechols can initiate toxicity through a diverse range of molecular mechanisms, ranging from direct protein damage to the production of reactive oxygen species (Schweigert *et al.*, 2001a; Schweigert *et al.*, 2001b). Despite this marked toxicity, the production of catechols appears to be a "necessary evil" in aromatic degradation. Indeed, catechol-producing oxygenases are among the most conserved enzymes in aromatic degradation pathways, likely due to the requirement for ring destabilization prior to cleavage and the abundance of molecular oxygen. Since the production of catechol is not easily avoided, bacteria have developed multiple strategies to minimize C23O inactivation and alleviate deleterious effects. Previously described strategies include the expression of "repair"enzymes (Cerdan et al., 1994; Hugo et al., 1998; Hugo et al., 2000; Park et al., 2002; Polissi and Harayama, 1993), the selection for, or recruitment of

inactivation-resistant C23Os (Cerdan *et al.*, 1994; Ramos *et al.*, 1987; Rojo *et al.*, 1987), and the selection of gene duplications which increase catechol consumption (Perez-Pantoja *et al.*, 2003). Additionally, alterations in the kinetic properties of key enzymes including a recently reported "less is more" strategy (George 2010) can also dramatically affect the range of aromatic substrates that individual bacteria can use for growth. Specific examples of these adaptations are discussed in the remainder of this review.

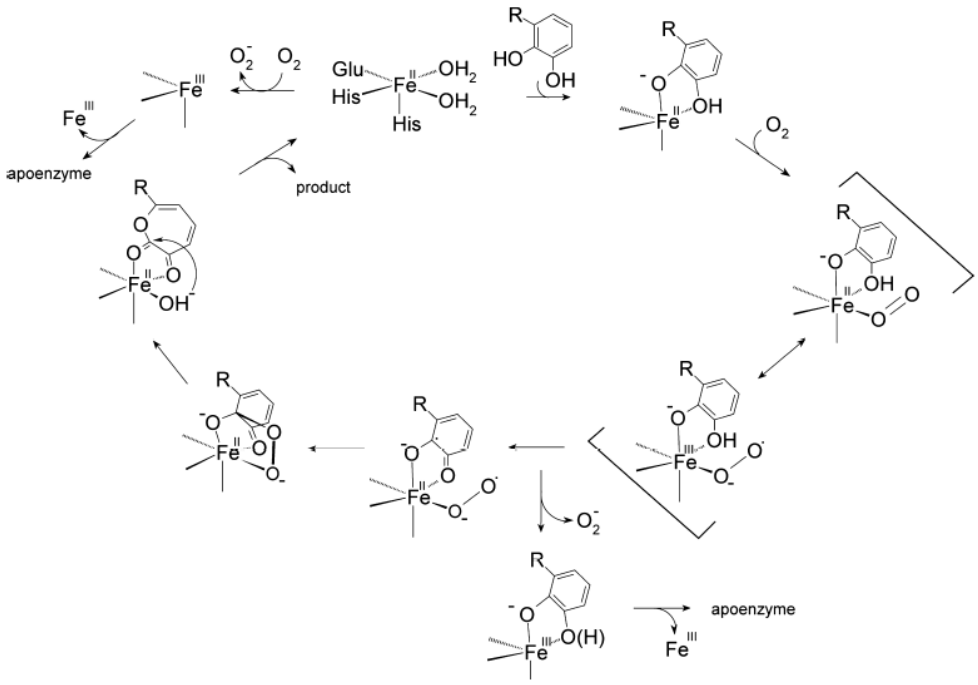


Figure 1.5. Catalytic mechanism and suicide inhibition of extradiol dioxygenases (C23Os). Inactivation results from the oxidation of active site Fe (II) to Fe (III). From (Vaillancourt *et al.*, 2006).

D. GENETIC ORGANIZATION AND REGULATION

Genes encoding aromatic degradation pathways are typically arranged in operons or clusters. These clusters are usually comprised of catabolic genes which encode degradative enzymes, transport genes encoding proteins which permit uptake of the compound, and one or more regulatory genes which control total gene expression (Diaz, 2004; Khomenkov *et al.*, 2008). The organization of a typical catabolic operon, encoding the toluene degradation (*tod*) pathway of *Pseudomonas putida* F1 (Zylstra and Gibson, 1989; Zylstra *et al*., 1988), is depicted in **Figure 1.6.** Catabolic gene clusters are often present on plasmids which facilitate horizontal gene transfer between hosts. Even when encoded on chromosomal DNA, genes associated with aromatic degradation are frequently flanked by mobile genetic elements or located within large transposons (Top and Springael, 2003; van der Meer et al., 1992). These mobile elements can facilitate rearrangement not only within the genome, but also between hosts through association with other mobile elements such as plasmids.

Proper functioning of a pathway is dependent not only on the possession of appropriate catabolic enzymes, but also on regulatory proteins that modulate gene expression in the presence of the suitable substrates. In catabolic pathways, regulatory systems generally fall into discrete families (Tropel and van der Meer, 2004). LysR-type regulators, for instance, comprise the largest family of bacterial regulatory proteins and function in the degradation of numerous aromatic compounds such as naphthalene and chlorobenzene. Other families include the IclR family, the XylR/NtrC family, and the AraC/XylS family, among others (Tropel and van der Meer, 2004). Extensive reviews of transcriptional regulators in aromatic degradation pathways have been published elsewhere and are beyond the scope of this review (Diaz and Prieto, 2000; Tropel and van der Meer, 2004). Intriguingly, closely related catabolic genes in distinct microorganisms are often regulated by different classes of

regulators, suggesting that the construction of a catabolic operon and the acquisition of transcriptional control are independent events (Cases and de Lorenzo, 2001; Shingler, 2003).

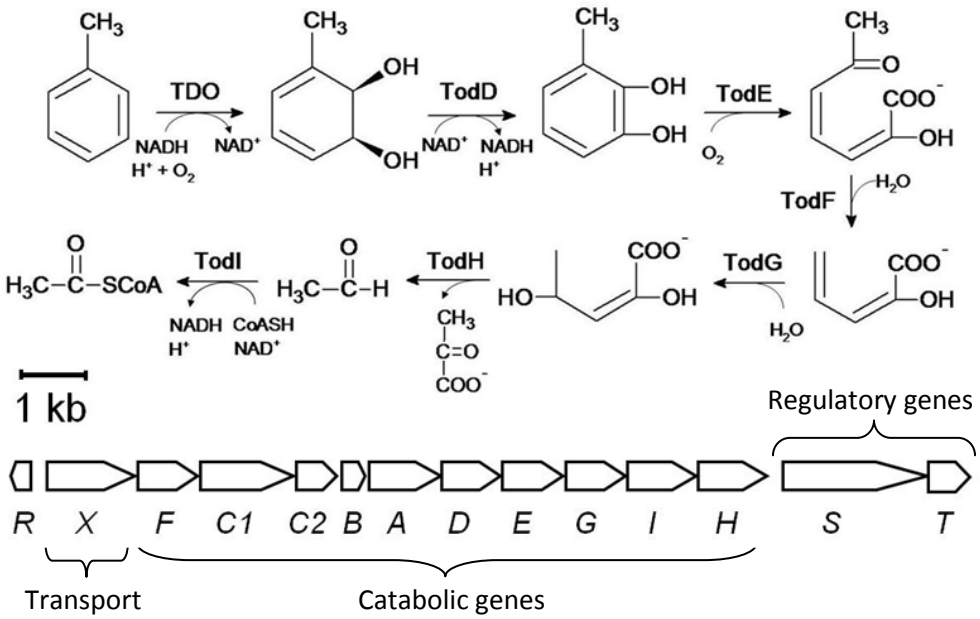


Figure 1.6. **Degradation pathway and genetic organization of the *tod* operon**. The degradation of toluene is pictured. The *todXFC1C2BADEGIH* operon is transcribed from a single promoter, P_{todX}. Transcriptional activation is mediated by a two-component system (TodS and TodT), encoded by genes located downstream of the catabolic operon.

III. BACTERIAL STRATEGIES FOR GROWTH ON AROMATIC COMPOUNDS

A. RECRUITMENT OF CATABOLIC GENES

The recruitment of alternative catabolic genes is perhaps the most widely-reported bacterial strategy for adapting to growth on aromatic compounds. Gene recruitment occurs in two different ways: vertical, or recruitment of catabolic function(s) from within a bacterium's genome, and horizontal, or recruitment involving gene transfer between bacteria.

1. VERTICAL RECRUITMENT

The vertical recruitment of catabolic function utilizes a bacterium's endogenous genome. While vertical recruitment is not capable of the sort of large-scale expansion of catabolic genes which characterize horizontal transfer, it can nevertheless have profound effects on bacterial adaptation and can even augment the effects of horizontal transfer. Strategies discussed below include changes in regulatory function, the spontaneous duplication of genes or clusters of genes, and the rearrangement or shuffling of pre-existing genes.

a. **Changes in regulation**

Mutations which result in changes in regulation are among the simplest adaptations which expand growth substrate range for aromatic compounds. In certain instances, aromatic substrates may not be degraded because transcriptional regulators do not recognize them as effectors. In these cases, even though cells possess the genetic capability to use the compounds for growth, the required catabolic proteins are not expressed. Mutations in regulatory proteins which alter effector specificity can

cause pathway induction and result in growth substrate expansion. In pathways regulated by repressors, point mutations can prevent repressor binding, resulting in constitutive expression of requisite proteins. In rare occurrences, mutations can create promoter binding sites, resulting in increased transcription and expression of catabolic genes. Insertion sequence elements (IS elements) are abundant in bacterial genomes (Mahillon and Chandler, 1998; Siguier et al., 2006) and have also been found to modulate regulatory function by increasing transcription of downstream genes (van der Meer, 2002).

Pseudomonas azelaica HBP1, for example, is an organism capable of growing on 2-hydroxybiphenyl (Kohler *et al.*, 1988) and encodes enzymes which are capable of mineralizing substituted phenols such as isopropylphenol. Despite this catabolic potential, HBP1 cannot grow on substituted phenols as sole carbon sources because HbpR, the positive regulator of the *hbp* pathway, is unable to recognize them as effectors (Jaspers *et al.*, 2000). When exposed to isopropylphenol for long periods of time, however, HBP1 mutants were generated which could grow on substituted phenols. In these adapted strains, mutations in *hbpR* expanded the range of effectors recognized by the regulator, resulting in transcription and growth on substituted phenols (Jaspers *et al.*, 2000; Kohler *et al.*, 1993). In *Pseudomonas* sp. CF600, mutations in the regulator *dmpR* were similarly capable of broadening effector specificity (Pavel *et al.*, 1994) or conferring constitutive phenotypes (Shingler and Pavel, 1995). An abundance of work on transcriptional control within the TOL plasmid of *Pseudomonas putida* has revealed analogous mutations in the regulators XylR and XylS (Dominguez-Cuevas *et al.*, 2008; Ramos *et al.*, 1997). In one study, it was found that single amino acid substitutions in the signal receptor domain of XylR conferred constitutive expression of TOL genes in the absence of an effector (Delgado *et al.*, 1995).

Other reports have demonstrated that mutations in the positive regulator XylS can lead to either constitutive phenotypes (Zhou *et al.*, 1990) or changes in effector specificity (Ramos *et al.*, 1986) and thereby expand growth substrate range. *Comamonas testosteroi* TA441 contains a silent cluster of genes (*aph*KLMNOPQB) encoding the catabolic enzymes phenol hydroxylase and catechol 2,3-dioxygenase, along with *aph*R, a divergently transcribed regulatory gene (Arai *et al.*, 1999b). In this pathway, it was found that mutations in *aphS*, a gene encoding a negative transcriptional regulator, derepressed transcription of the *aph* genes and conferred growth on phenol (Arai *et al.*, 1999a).

In certain degradation systems, multiple regulatory mutations are required to confer growth on new aromatic substrates. In *Pseudomonas putida* F1 and related strains, transcription of the toluene degradation (*tod*) pathway (Zylstra and Gibson, 1989; Zylstra et al., 1988) is regulated by the two-component system TodST (Lau *et al.*, 1997). TodS, the sensor kinase, recognizes a wide range of effectors (Busch *et al.*, 2007; Lacal *et al.*, 2006) and phosphorylates TodT (Lacal *et al.*, 2008), the response regulator responsible for binding the *tod* promoter and activating transcription. Choi *et* al. (2003) demonstrated expansion of growth substrate range in F1 to include *n*-propylbenzene, *n*-butylbenzene, cumene, and biphenyl following two separate mutations in regulatory function. Mutations in *todS* expanded the range of effectors capable of activating transcription of the *tod* genes, while a mutation in *cymR*, a repressor of the cumate (*cmt*) operon (Eaton, 1996; Eaton, 1997), allowed for constitutive expression and recruitment of the broad substrate MFP hydrolase CmtE (Choi *et al.*, 2003). In wild-type F1, *meta*-fission products accumulated due to the inability of TodF, the endogenous MFP hydrolase of the *tod* pathway, to degrade them. Impaired CymR binding also allowed for expanded growth substrate range in *Pseudomonas putida* CE2010, although this was achieved through a mutation in the

cmt promoter rather than a mutation in the repressor itself (Ohta *et al.*, 2001). In *Pseudomonas putida* KL47, a strain capable of growing on diverse substrates such as biphenyl and cumate, mutation of TodS was identified as a crucial factor in catabolic adaptation. Although, once again, CymR binding was impaired, allowing constitutive expression of the cumate operon and the broad-substrate MFP hydrolase CmtE as well (Lee *et al.*, 2006).

In rare instances, mutations can create consensus promoters upstream of catabolic genes. Using a promoterless phenol degradation operon (*phe*BA) in *Pseudomonas putida*, it was shown that exposure to phenol generated mutants capable of growth (Phe +). Further analysis of Phe+ mutants revealed that specific base substitutions were predominant in phenol-exposed cells and resulted in the formation of a sequence similar to the consensus σ70 promoter (Kasak *et al.*, 1997). It was also found that the introduction of an IS elements could result in the creation of a new promoter and generate Phe+ mutants. Some IS elements, such as IS1411 (Kallastu *et al.*, 1998), contain sequences near their termini which function as constitutive promoters. When these IS elements insert upstream of catabolic operons, constitutive transcription may take place. Working with promoterless *pheBA* genes expressed in *Pseudomonas putida* PaW85 (Kasak *et al.*, 1993), it was found that some spontaneous Phe+ mutants had IS1411 inserted upstream of *pheB* (Kallastu *et al.*, 1998). Since IS1411 contains a strong promoter at its 3' end, its integration upstream of *pheBA* drove transcription and conferred the ability to grow on phenol. An earlier study also found that transposon integration upstream of *pheBA*, combined with point mutations in the promoter permitted *P. putida* to grow on phenol (Nurk *et al.*, 1993). In addition to creating new promoters, IS elements are also capable of modulating transcription through interruption of genes encoding repressors or their binding sites as discussed above.

b. **Altering gene dosage**

Through the action of genetic engineering, homologous recombination, or transposition, the copy number of genes or clusters of genes may be increased. Duplications have long been considered an essential mechanism in the evolution of xenobiotic-degradation pathways (van der Meer *et al.*, 1992). Once a gene has been duplicated, the sequence of the redundant gene copy may diverge and accumulate mutations at an increased rate. As a result of mutation, substrate range or catalytic efficiency may be drastically altered (Section III. C.). In some cases, this may expand the range of aromatic substrates available for growth.

Duplications of genes have been frequently observed in aromatic degradation pathways. In pathways which produce toxic intermediates such as chlorocatechols, specific catabolic gene duplications appear to serve novel roles in the mitigation of toxicity. *Ralstonia eutropha* JMP134(pJP4) (formerly known as *Alcaligenes eutrophus*), originally isolated for its ability to metabolize 2,4-dichlorophenoxyacetic acid, is also capable of growing on 3-chlorobenzoate through the *tfd* pathway. Plasmid pJP4 includes two apparently isofunctional catabolic gene clusters, $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ (Laemmli *et al.*, 2000; Trefault *et al.*, 2004), which encode proteins involved in chlorocatechol metabolism. Of particular importance is chlorocatechol-1,2-dioxygenase (TfdC), the enzyme responsible for *ortho*-ring cleavage. Intriguingly, it was found that multiple copies of *tfdC* were required for *Ralstona eutropha* JMP(pJP4) to grow on 3-chlorobenzoate (Perez-Pantoja *et al.*, 2003). In mutants which possessed only one *tfdC* gene, 3-chlorocatechol accumulated and caused marked toxicity. Thus, increasing the gene dosage of *tfdC* through duplication augmented the rate of chlorocatechol turnover, preventing toxicity and expanding growth substrate range. Duplications of chlorocatechol dioxygenase genes were also observed in the *clc*-element of *Ralstonia* sp. strain JS705 and might play

similar roles (Muller et al., 2003; van der Meer and Sentchilo, 2003; van der Meer et al., 1998).

In the laboratory, gene dosage can be increased through the expression of plasmid-borne catabolic genes. In *P. putida* F1, for instance, a construct which over-expressed TodE, the endogenous C23O of the *tod* operon, was capable of growth on styrene while the wild-type strain was not (George *et al.*, 2010). Expression of TodE in *trans* mimicked the effect of gene duplication and enhanced the rate of 3-vinylcatechol turnover, preventing toxicity in a similar fashion as *Ralstonia eutropha* JMP(pJP4).

Since duplications create homologous regions of DNA, they may direct catabolically-relevant recombination mechanisms. If present on a plasmid, for instance, these duplications can cause integration of catabolic genes into the chromosome. This appears to be the case in the TOL plasmid, where two direct repeats have been suggested to be necessary for integration of this catabolic operon into the chromosome (Meulien *et al.*, 1981; Sinclair *et al.*, 1986). Thus, in addition to increasing gene dosage and the amount of critical enzymes, duplications can also play essential roles in the dissemination of catabolic genes through horizontal transfer (Section III. A. 2). Similar gene repeats have been found in numerous other catabolic plasmids and may similarly allow for recombination into or out of chromosomal DNA (van der Meer, 2002).

c. **Genetic rearrangement and gene capture**

In nearly every described catabolic pathway, there is clear evidence that gene rearrangements have occurred (van der Meer, 2002). Transposons or IS elements are located within or in close proximity to many catabolic genes, suggesting that they have played a role in DNA shuffling and the movement of genes throughout the host's

genome. In *Pseudomonas sp.* strain P51, for instance, the *tcbA* and *tcbB* genes for chlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase are located on a transposable element, Tn5280 (van der Meer *et al.*, 1991). Tn5280 appeared to function as a composite transposon (Top and Springael, 2003), with nearly identical IS elements (IS1066 and IS1067) located at each end. The structure was mobile, and it was suggested that these IS elements had captured the *tcaAB* genes from another organism – likely with the aid of an additional mobile element (see Horizontal Recruitment, Section III.A.2 below) – and subsequently grouped them with pre-existing genes for chlorocatechol metabolism (van der Meer *et al.*, 1991; Werlen *et al.*, 1996). This interplay between horizontal transfer and vertical rearrangement is typical in aromatic degradation pathways. It is evident that similar events have occurred in numerous other organisms and played crucial roles in pathway evolution. A number of additional examples involving gene rearrangement in catabolic pathways have been reviewed elsewhere (Khomenkov et al., 2008; Springael and Top, 2004; Top and Springael, 2003).

As previously noted (Section II. D.), catabolic pathways tend to be organized into gene clusters which are under the control of one or more regulatory proteins (Tropel and van der Meer, 2004). Analysis of flanking regions makes it clear that IS elements and other mobile elements are responsible for the majority of this organization (Khomenkov *et al.*, 2008). The clustering of catabolic genes into upper and lower pathways, along with the recruitment of regulatory control, is important for the assembly of a contiguous transcriptional unit and thus the energetically efficient degradation of aromatic compounds (van der Meer, 2002).

Although pathway genes are usually grouped into one or two transcriptional units, this is not always the case. In the dibenzodioxin and dibenzofuran degrader *Sphingomonas* sp. strain RW1, for instance, catabolic genes are scattered throughout

the genome (Armengaud *et al.*, 1998). Intriguingly, while expression of upper pathway genes is modulated according to the presence of a suitable carbon source, expression of *dbfB*, the ring cleavage dioxygenase, is constitutive. This sort of differential expression is unusual and contrasts with many other well-studied catabolic pathways. In the course of analysis, numerous other catabolic genes were discovered dispersed throughout the genome (Armengaud *et al.*, 1998). This scattering of diverse catabolic genes has been observed in other *Sphingomonas* strains such as *Sphingomonas yanoikuyae* B1(Kim and Zylstra, 1999) and appears to be consistent with the catabolic diversity of this genus: although energetically expensive in some environments, a lack of coordinate regulation and constitutive expression of certain catabolic gene modules may aid in the utilization of varied aromatic substrates for growth.

2. HORIZONTAL RECRUITMENT

Horizontal recruitment of catabolic genes is mediated by a variety of mobile genetic elements which comprise a "horizontal gene pool" (Thomas 2000). Elements capable of horizontal transfer include plasmids, transposons, and "genomic islands," all of which are capable of transferring catabolic genes between disparate bacterial hosts. This transfer allows bacteria to rapidly adapt to, and degrade, aromatic compounds – even those compounds which are "newcomers" to the global environment (Top and Springael, 2003; van der Meer and Sentchilo, 2003). Although gene recruitment through plasmid transfer and transposition are discussed separately below, these elements often work in tandem *in vivo*. For instance, many plasmid-encoded catabolic pathways such as the TOL catabolic operon are flanked by insertion sequences or occur within transposable elements.

a. **Plasmid transfer**

In aromatic degradation, the importance of plasmid-encoded catabolic genes cannot be overstated. The transfer of a plasmid is a one-step process which can result in the acquisition of up to 250 kb of DNA or more and can drastically increase catabolic capacity. Natural plasmid transfer – combined with the potential for mutation – creates enormous genetic potential in aromatic-degrading bacteria. The fact that some plasmids are capable of *retrotransfer*, or the capture of chromosomally-encoded catabolic genes, adds to this tremendous potential (Ronchel *et al.*, 2000; Trefault *et al.*, 2004). Nearly every catabolic pathway described in the literature, even those that are not plasmid-encoded, is associated with the presence of plasmids (van der Meer, 2002). Perhaps the most well studied example is the TOL plasmid, encoding a pathway for the degradation of toluene and related compounds (Worsey and Williams, 1975). Plasmids involved in the degradation of naphthalene, xylenes, chlorobenzenes, and atrazine (among many others) have also been described (Cao *et al.*, 2009; Dennis, 2005). The majority of these plasmids are transmissible and there is strong evidence for widespread natural plasmid transfer *in situ* (Dejonghe et al., 2000; Herrick et al., 1997; Peters et al., 1997; Springael and Top, 2004; Top et al., 2002). For example, phenol-degrading *Pseudomonas putida* PaW85, containing plasmid-encoded *pheAB* genes, was deliberately released into a mine water-contaminated river. Several years following this release, it was found that approximately one-third of phenol-degrading bacteria isolated from the site contained the plasmid-encoded *pheAB* genes. Intriguingly, these bacteria were all different species than the original donor strain, strongly suggesting horizontal gene transfer (Peters *et al.*, 1997). More recently, a series of studies utilized 2,4-dichlorophenoxyacteic acid (2,4-D) as a model compound and two plasmids which encoded 2,4-D degradation genes to study catabolic plasmid transfer in soil. A correlation between plasmid transfer to

indigenous bacteria and augmented 2,4-D degradation was demonstrated in certain cases, emphasizing the efficacy of natural plasmid-transfer, even when the original donor may not thrive in the new environment (Newby et al., 2000a; Newby et al., 2000b; Newby and Pepper, 2002).

The utilization of catabolic plasmid transfer to create hybrid strains capable of degrading an expanded range of aromatics has been reviewed extensively (Cao *et al.*, 2009; Reineke, 1998; van der Meer *et al.*, 1992). In many cases, plasmid-encoded genes are able to overcome metabolic blocks, allowing for complete degradation of a previously recalcitrant compound. This is generally known as the "patchwork" approach, and combines enzymes from two or more existing pathways (Copley, 2000). The patchwork approach has been effectively utilized to produce strains capable of degrading a variety of recalcitrant aromatic compounds. To degrade monochlorobiphenyls, the *ohb* operon of *Pseudomonas aeruginosa* and the *fcb* operon of *Arthrobacter globiformis* was cloned and expressed in *Comamonas testosterone* sp. VP44 (Hrywna *et al.*, 1999). Monti *et al*. introduced genes involved in the degradation of 2,4-dinitrotoluene from *Burkholderia* sp. DNT into *Pseudomonas fluorescens* ATCC 17400, allowing for complete mineralization of the compound (Monti *et al.*, 2005). Through transfer of the well-studied TOL plasmid pWWO, recombinant *P. putida* F38/D has been constructed which combines the *tod* and TOL pathways to degrade benzene, toluene, and *p*-xylene (Lee *et al.*, 1994). A follow-up study created the hybrid strain *P. putida* TB105 which was also capable of degrading benzene, toluene, and *p*-xylene, this time through introduction of a plasmid which expressed *tod* pathway genes to augment the host-encoded TOL pathway (Lee *et al.*, 1995). Analysis of natural isolates also makes it clear that a patchwork approach using plasmid-encoded genes is a broadly effective strategy for adaptation to aromatic compounds (Copley, 2000; Top and Springael, 2003).

b. **Conjugative transposons and genomic islands**

Although class I and class II transposons (Springael and Top, 2004; Top and Springael, 2003) can move within a single host's genome, they are typically incapable of horizontal transfer without the aid of a plasmid. Conjugative transposons are distinct from other classes in their ability to excise from host DNA, form a circular intermediate, and integrate into a new DNA target. This capability allows conjugative transposons to move laterally between hosts without the aid of plasmids (Tsuda *et al.*, 1999). As with plasmid transfer, integration of conjugative transposons can rapidly expand the catabolic repertoire of the target bacterium. In the literature, there are several examples of catabolic genes associated with conjugative transposons. In *Ralstonia oxalatica* A5, a strain capable of metabolizing biphenyl and 4-chlorobiphenyl, the 13-kb *bph* region is located within a 55-kb transposon, Tn*4371* (Merlin et al., 1997; Merlin et al., 1999). Tn*4371*-like catabolic sequences have since been identified in other (chloro-)biphenyl-degrading bacteria and appear to encode expanded degradative capacity (Merlin *et al.*, 1997; Springael *et al.*, 2001). An additional example of a conjugative catabolic transposon is the *bph-sal* element of *P. putida* strain KF715. The *bph-sal* element encodes genes for biphenyl and salicylate metabolism and is located on a 90 kb chromosomal fragment. This element can be transferred to different *P. putida* strains – without the aid of a plasmid – at frequencies of approximately 1×10^{-6} per recipient cell (Nishi *et al.*, 2000).

Although they may be technically classified as conjugative transposons, large, self-transmissible regions of DNA such as Tn*4371* contain specific features which allow them to be considered 'genomic islands' (Ravatn et al., 1998; van der Meer and Sentchilo, 2003). In relation to aromatic degradation, the term 'genomic island' has been proposed for mobile regions of DNA, typically carrying genes for integration and conjugation, which encode catabolic proteins. As in the spread of pathogenic or

antibiotic resistance 'islands', the transfer of catabolic genomic islands provides bacteria with a dynamic genetic resource for rapid adaptation and evolution (Juhas *et al.*, 2009). Perhaps the best known example is the *clc*-element which was originally identified in the 3-chlorobenzoate-degrading organism *Pseudomonas* sp. B13 (Gaillard *et al.*, 2006; van der Meer *et al.*, 2001). The *clc*-element comprises a 105 kb region of DNA which carries the *clcRABD* gene cluster. These genes are involved in the mineralization of chlorobenzoate and chlorocatechols via *ortho*-cleavage. In a variety of bacteria, the *clc*-element integrates site-specifically into a tRNA gene, mediated by the P4-type integrase IntB13. Similar elements which carry identical catabolic genes exist in other chlorobenzoate-degrading bacteria which were independently isolated from geographically distinct locations (Gaillard *et al.*, 2006). Interestingly, the *clc*-element has also been shown to undergo duplication events which were necessary for its benzene-degrading host to grow on chlorinated benzenes (van der Meer *et al.*, 1998). This example further demonstrates the intertwining of both vertical and horizontal strategies for enhancing growth substrate range. Genomic islands related to the *clc*-element, but carrying different catabolic information such as that for biphenyl degradation, have also been described (van der Meer and Sentchilo, 2003).

B. EXPRESSION OF REPAIR ENZYMES

The expression of proper catabolic genes does not always ensure the efficient degradation of aromatic compounds. Indeed, aromatic compounds and their degradation byproducts can cause enzyme inactivation and, in some cases, lead to more generalized cellular toxicity. To deal with these deleterious effects, bacteria may express proteins which alleviate toxicity and allow for proficient aromatic metabolism. Below, we discuss two classes of proteins: those involved in enzyme reactivation, and those which alleviate general cellular stress.

1. ENZYME REACTIVATION

As mentioned above (Section II. C.), metabolites formed during aromatic degradation have the capacity to inactivate catabolic enzymes. In the case of catecholic intermediates, inactivation of C23Os may occur directly during ring cleavage, in a process known as suicide inhibition (Bartels et al., 1984; Klecka and Gibson, 1981), or indirectly through the action of oxidative species (Vaillancourt *et al.*, 2006). Suicide inactivation has been shown to limit the range of toluates degraded by the TOL pathway, polychlorinated biphenyls degraded by the *bph* pathway, and chloro- and alkyl-benzenes degraded by the *tod* pathway (Cerdan et al., 1994; Klecka and Gibson, 1981; Ramos et al., 1987; Rojo et al., 1987; Vaillancourt et al., 2002; Ward et al., 2004).

Given the crucial role of C23O inactivation in limiting substrate range, some bacteria have developed enyzmatic "repair" systems to reverse enzyme inactivation. Collectively, these proteins are known as [2Fe-2S] plant-like ferredoxins. Plant-like ferredoxins catalyze a redox-dependent reaction, reducing oxidized Fe(III) to the catalytically active Fe (II) (Hugo *et al.*, 1998). Perhaps the most well-studied [2Fe-2S] ferredoxin is XylT, which promotes reactivation of XylE, a C23O in the TOL plasmid-encoded pathway of *Pseudomonas putida* mt2. Mutants deprived of a functional *xylT* gene lost the ability to grow on both *p*-xylene and *p*-toluate as sole carbon sources (Polissi and Harayama, 1993). The impeded growth of *xylT* mutants, coupled with the observation that the intermediate 4-methylcatechol irreversibly inactivated XylE only in mutant strains, led to the supposition that XylT functioned in C23O reactivation (Polissi and Harayama, 1993). Subsequent purification of XylT revealed that it was a [2Fe-2S] ferredoxin which reactivated XylE through reduction of the iron atom in the enzyme active site (Hugo *et al.*, 1998). Further studies have revealed the existence of XylT-like [2Fe-2S] ferredoxins in other catabolic pathways

including those for the degradation of naphthalene, cresols, nitrobenzene, aniline, and chlorobenzene (Herrmann et al., 1995; Hugo et al., 2000; Parales et al., 1997; Powlowski and Shingler, 1994; Tropel et al., 2002). The abundance of [2Fe-2S] ferredoxins in such a diverse array of pathways and bacteria underscores the efficacy of this particular strategy of substrate expansion. Through this reactivation strategy, bacteria are able to increase catechol turnover and permit growth on previously inaccessible substrates. Given catechols' noted toxicity (Section II. C), efficient catechol turnover is essential for the viability of cells producing it. Recently, it was shown that expression of the C23O DmpB, along with its endogenous ferredoxin DmpQ, reduced C23O inactivation and allowed *P. putida* F1 to acquire the ability to grow on styrene (George *et al.*, 2010). Intriguingly, expression of DmpQ alone also protected against cell death, preventing marked declines in cell viability observed in wild-type F1 exposed to styrene, even though its expression was not sufficient to confer growth on styrene.

2. REDUCTION OF CELLULAR STRESS

In addition to inactivating the enzymes responsible for their degradation, catechols can also exert toxicity directly through a variety of mechanisms such as membrane stress and redox cycling (Schweigert *et al.*, 2001a; Schweigert *et al.*, 2001b). Catechols, however, are not the only intermediates which may be acutely toxic. Various hydroxylated intermediates and products of ring-fission may also be toxic to bacterial cells. In addition to direct metabolite toxicity, evidence has accumulated which suggests bacteria experience considerable oxidative stress during the degradation of many aromatic compounds (Imlay, 2003). During the degradation of polychlorinated biphenyls, for example, metabolism is accompanied by the production of reactive oxygen species and the induction of cellular stress (Chavez *et*

al., 2006). In some instances, the parent aromatic compound is also capable of exerting toxicity. This is the case for many hydrophobic polycyclic aromatic hydrocarbons as well as solvents such as toluene and styrene (Ramos *et al.*, 2002). Growth in the presence of high concentrations of toluene and similar aromatic solvents is enhanced, for example, by the expression of efflux pumps or by alterations in membrane fluidity (Ramos *et al.*, 2002). Importantly, one of the efflux pumps (TtgGHI) is plasmid encoded, further demonstrating the importance of horizontal gene transfer in adaption to growth on aromatic compounds (Rodríguez-Herva *et al.*, 2007).

The diversity of toxic insults accompanying aromatic exposure and degradation has required bacteria to develop numerous detoxification mechanisms. Some strains, for example, may utilize glutathione S transferases (GSTs) which are broadly involved in cellular detoxification against harmful xenobiotics and can protect against oxidative stress (Allocati *et al.*, 2009). With respect to the degradation of aromatic compounds, bacterial GSTs are involved in the degradation of monocyclic aromatics such as phenol (Santos *et al.*, 2002) as well as polycyclic aromatics such as anthracene (Kim *et al.*, 1997). A GST first characterized for its involvement in biphenyl degradation (Bartels *et al.*, 1999; Hofer *et al.*, 1994), BphK, is one of the most extensively studied bacterial GSTs and may have a role in multiple pathways (Allocati *et al.*, 2009). In *Sphingomonas yanoikuyae* B1, for instance, the presence of a *bphK* gene allows the organism to grow more efficiently on *m*-toluate (Bae *et al.*, 2003). Through expression of GSTs such as BphK, bacteria can achieve efficient degradation of aromatic compounds that would otherwise preclude growth due to toxicity. More detail on GSTs and their role in aromatic degradation can be found in the extensive review of Allocati *et* al. (2009).

As with biphenyl, naphthalene has been reported to be toxic to some bacteria (Ahn et al., 1998; Garcia et al., 1998; Park and Madsen, 2004; Pumphrey and Madsen,

2007) and its metabolism can lead to the production of reactive oxygen species (ROS) (Kang *et al.*, 2006). Intriguingly, the addition of antioxidants such as ascorbate was found to reduce the inhibitory effects of ROS and increase biodegradation efficiency (Kang *et al.*, 2006). In a follow-up study, it was found that over-expression of antioxidant enzymes enhanced naphthalene biodegradation in *Pseudomonas* sp. strain AS1 (Kang *et al.*, 2007). Specifically, the authors found that expression of a ferredoxin-NADP+ reductase (Fpr) or superoxide dismutase (SOD) in tandem with alkyl hydroperoxide reductase (AhpC) conferred resistance to oxidative stress and simultaneously increased growth-rate. These results show that over-expression of antioxidant enzymes represents an effective bacterial strategy for enhancing biodegradation and are consistent with a proteomic analysis of *Pseudomonas putida* KT2440 which demonstrated that the production of proteins involved in oxidative stress defense such SOD and AhpC increased following pollutant exposure (Santos *et al.*, 2004).

C. DIRECT MODULATION OF SUBSTRATE RANGE OR KINETIC PARAMETERS

While changes in enzyme substrate range or kinetics seem trivial – especially when compared to large scale vertical gene duplications or horizontal transfer – they can play an important role in the development of new catabolic functions. These changes are usually the result of errors in DNA replication and repair systems, which occur at a low yet detectable rate (Kunkel, 2004). The low mutation rate is not constant, however, and can increase due to the induction of error-prone polymerases under stressed conditions (Bull *et al.*, 2001; Foster, 2007; Goodman, 2002; Nohmi, 2006; Tegova *et al.*, 2004), the formation of sequence-dependent secondary structures (Gerischer and Ornston, 1995; Hartnett et al., 1990; Wright, 2000), or the transient

formation of single stranded DNA during transcription (Wright, 2000). Point mutations, insertions, and small deletions formed during DNA replication and repair have been observed in nearly every aromatic catabolic pathway (van der Meer, 2002). The effects of these mutations on the properties of catabolic enzymes is generally well-understood and several examples are discussed below.

1. INCREASED EFFICIENCY OR SUBSTRATE RANGE

In genes encoding catabolic enzymes for aromatic degradation, relatively simple base pair mutations are capable of altering substrate specificity or kinetic parameters (van der Meer *et al.*, 1992). These subtle changes can have profound effects on biodegradation efficiency and, in some cases, result in expansion of growth substrate range. In laboratory systems, spontaneous modification of enzyme kinetic properties or substrate specificity has led to growth on new aromatic substrates. In the TOL plasmid pWWO of *P. putida* mt-2, cleavage of 4-ethylcatechol, an intermediate in 4-ethylbenzoate metabolism, results in suicide inhibition of the C23O XylE. When selected on 4-ethylbenzoate, however, spontaneous mutants were isolated with substitutions in XylE which rendered it resistant to inhibition by 4-ethylcatechol and in turn allowed mt-2 to grow on 4-ethylbenzoate (Ramos *et al.*, 1987). A later study further characterized two spontaneous C23O mutants resistant to 4-ethylcatechol-mediated inactivation and developed a quantitative relationship between C23O inactivation resistance and cell growth rate (Cerdan *et al.*, 1994). In other pathways subject to C23O inhibition such as that for biphenyl degradation (bph), similar mutations have produced ring cleaving dioxygenases resistant to suicide inactivation by the relevant catechols (Vaillancourt *et al.*, 2005). This expansion of growth substrate specificity by simple base pair mutations was also observed in the TOL plasmid upper pathway. Mutants were isolated which contained substitutions in

toluene oxidase which expanded the substrate range to include *p*-ethyltoluene (Abril *et al.*, 1989). Additional examples of mutations which expand substrate range by increasing the rate of substrate transformation are common in the scientific literature and have been reviewed elsewhere (Khomenkov *et al.*, 2008; van der Meer *et al.*, 1992).

In recent years, there has been considerable interest in the directed evolution of catabolic proteins for use in bioremediation or biocatalysis (Diaz, 2004; Timmis and Pieper, 1999; Urgun-Demirtas et al., 2006; Yuan et al., 2005). Much of this work has been based on information gathered from spontaneous mutants as described above. Using a variety of techniques, significant progress has been made in the generation of catalytically improved biphenyl (Furukawa *et al.*, 2004; Kumamaru *et al.*, 1998; Suenaga *et al.*, 2001), naphthalene (Fortin et al., 2005; Gibson and Parales, 2000; Parales et al., 1998), and dioxin (Furukawa, 2000) dioxygenases. Other catabolic proteins involved in aromatic degradation such as C23Os have also been successfully engineered with improved catalytic efficiency or broadened substrate range (Cao *et al.*, 2009).

2. CATABOLIC ENZYME INACTIVATION

If the activity of a particular enzyme is inhibitory to growth, studies have shown that the isolation of mutants with abolished enzyme activity is readily accomplished. This has been clearly illustrated in cases where normal enzyme function produces a toxic metabolite or results in pathway misrouting. In the metabolism of *p*-hydroxybenzoate by *Acinetobacter calcoaceticus*, for example, protocatechuate 3,4-dioxygenase (PcaGH) generates the toxic intermediate β-carboxymuconate. When an *A. calcoaceticus* strain was exposed to physiological conditions where expression of PcaGH inhibited growth, Gerischer and Ornston

(1995) were able to isolate numerous mutants where base substitutions in *pcaG* or *pcaH* eliminated proteocatechuate-3,4-dioxygenase activity. These mutants failed to cleave protocatechuate and thus did not accumulate β-carboxymuconate. In other isolates, abolition of protocatechuate 3,4-dioxygenase activity was achieved through lengthy deletions or gene interruption by IS1236, an insertion element. Specifically, the authors implicated DNA slippage structures and subsequent mispairing as the mechanism behind increased mutation rates (Gerischer and Ornston, 1995).

In natural isolates, there is also evidence for the inactivation of deleterious enzyme functions. *Pseudomonas sp.* P51, a strain capable of degrading chlorobenzene, contains a mosaic pathway with a chlorobenzene dioxygenase (encoded by *tcbA*) and a dehydrogenase (encoded by *tcbB*) related to enzymes involved in toluene (*todC1C2BAD*) or benzene degradation (*bedC1C2BA*) (Werlen *et al.*, 1996). A large gene fragment encoding a non-functional *meta*-cleavage dioxygenase homologous to *todE* was also found downstream of *tcbB.* The authors suggested that this gene fragment, along with one similar to *todF* located upstream of *tcbA*, were evolutionary remnants of the recombination event which eventually resulted in the capture of *tcbA* and *tcbB.* In chlorobenzene degradation, the *ortho*-cleavage pathway produces intermediates that can support growth while the *meta*-pathway is unproductive and typically induces toxicity (Werlen *et al.*, 1996). Since the *todE*-like gene was deleterious to the cell, it may have been necessary for the organisms to eliminate *meta*-cleavage activity to prevent pathway misrouting and toxicity. In another isolate capable of chlorobenzene degradation, *Burkholderia* sp. strain PS12, a gene encoding a *meta*-cleaving dioxygenase was similarly inactivated (Beil *et al.*, 1999). Intriguingly, the genomic context of the chlorobenzene degrading *tec* genes in strain PS12 was very similar to that of *Pseudomonas sp.* strain P51 in that it still retained *todF* and *todE*-like remnants. In this case, however, simple point

mutations inactivated the *meta*-cleavage enzyme rather than a deletion. Although strains PS12 and P51 used different molecular mechanisms, the general strategy of enzymatic inactivation proved equally effective. Inactivation of genes responsible for metabolite misrouting has also been observed in several other isolates, such as those involved in the degradation of nitroaromatic compounds (Ju and Parales, 2009).

3. "LESS IS MORE"

The efficient turnover of catechol intermediates in aromatic degradation pathways is critical due to their toxicity and unique ability to inactivate the dioxygenases responsible for their cleavage (Section II. C). Since catechol production is required for productive aromatic metabolism in many pathways, enzyme inactivation as described above is not always possible. Recently, a novel strategy for the reduction of catechol-induced toxicity was described in *Pseudomonas putida* F1, the model organism harboring the *tod* pathway.

P. putida F1 is unable to grow on styrene due to the deleterious effects of 3-vinylcatechol, an intermediate in degradation. When exposed to styrene, 3-vinylcatechol inactivates the C23O TodE and subsequently accumulates, causing marked reductions in cell viability (George *et al.,* 2010) (**Figure 1.7**). When styrene-naïve F1 cells were exposed to styrene as a sole carbon source, mutants were eventually isolated which did not accumulate 3-vinylcatechol and could grow on styrene (George 2010). Characterization of one mutant, designated SF1, revealed the presence of a single base pair mutation (C479T) in the reductase component (*todA*) of toluene dioxygenase (*todC1C2BA*), the enzyme responsible for the production of 3-vinylcatechol from styrene (George 2010). Further analysis revealed that this simple mutation resulted in attenuated toluene dioxygenase (TDO) activity and a decreased

rate of 3-vinylcatechol production. SF1's *todA* allele ($todA_{C479T}$) was then expressed in styrene-naïve F1 and found to be sufficient to permit growth on styrene, whereas expression of a wild type copy of *todA* in SF1, reduced growth on styrene and lead to 3-vinylcatechol accumulation. Unlike previously discussed strains that adapted to toxic catechol intermediates by developing resistant C23Os (Section III.C.1), SF1's C23O activity and substrate range remained unchanged.

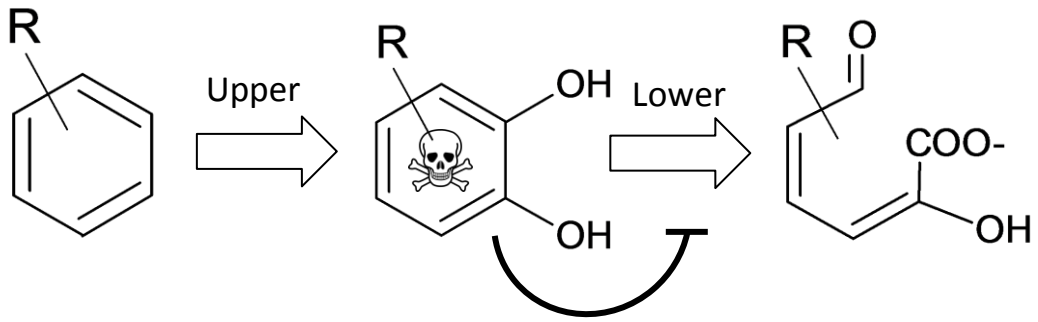
As this particular strategy relies on a decrease rather than an increase in enzyme activity, theoretically, a large number of mutations could have resulted in reduced catechol production. Indeed, characterization of four additional styrene-adapted mutants revealed the presence of reduced toluene dioxygenase activity in all strains despite the absence of the mutant *todA* allele of SF1 (George 2010).

The net effect of decreasing 3-vinylcatechol production via this decrease in TDO activity was similar to that observed when F1's C23O *todE* was over expressed (George *et al*., 2010): no 3-vinylcatechol accumulated and styrene supported growth. These observations are suggestive of an alternative "less is more" strategy and demonstrate that there multiple ways for cells to protect themselves from the accumulation of potential deleterious catechols (George 2010) (**Figure 1.8)**. Given the ubiquity of catechol intermediates in aromatic degradation, it is likely that this strategy for alleviating catechol toxicity may also be effective in other catabolic pathways.

Non-productive scenarios

Results: abolition of C23O activity, accumulation of catechol, toxicity, no growth on the parent substrate

(a) **Wild-type**



(b) **Increased operon transcription (single promoter)**

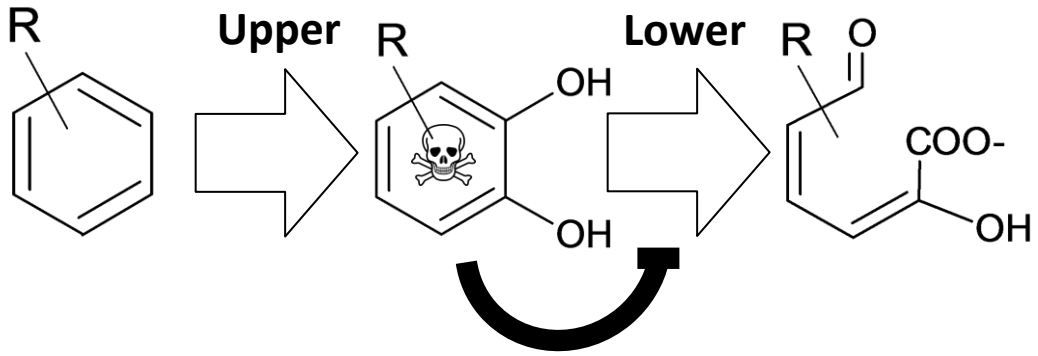
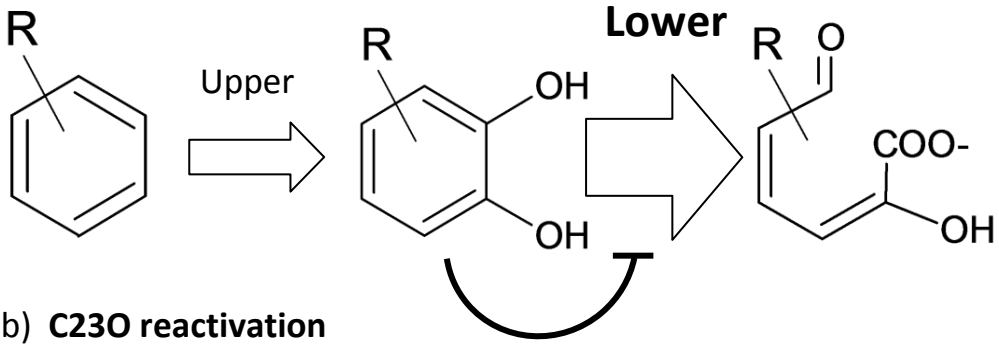


Figure 1.7. Non-productive scenarios for growth on substrates which produce C23O-inactivating catechols. (a) The rapid production of catechol by the upper pathway inactivates C23O activity (blocked curved line) and results in catechol accumulation and toxicity (Skull and bones) **(b)** Since the *tod* pathway (and many other catabolic pathways) are transcribed from a single promoter, upregulating transcription may have no effect on net catechol turnover. Increased upper pathway activity produces greater amounts of catechol and amplifies C23O inactivation, negating the effect of increased C23O activity (thicker curved line).

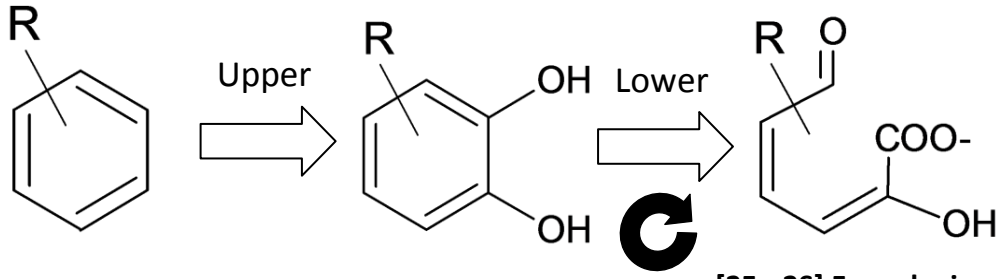
Productive scenarios

C23O activity is retained, preventing the accumulation of catechol and conferring growth on the parent substrate

(a) **Selective enhancement of lower pathway**



(b) **C23O reactivation**



[2Fe-2S] Ferredoxin reactivates oxidized C23O

(c) **"Less is more"**

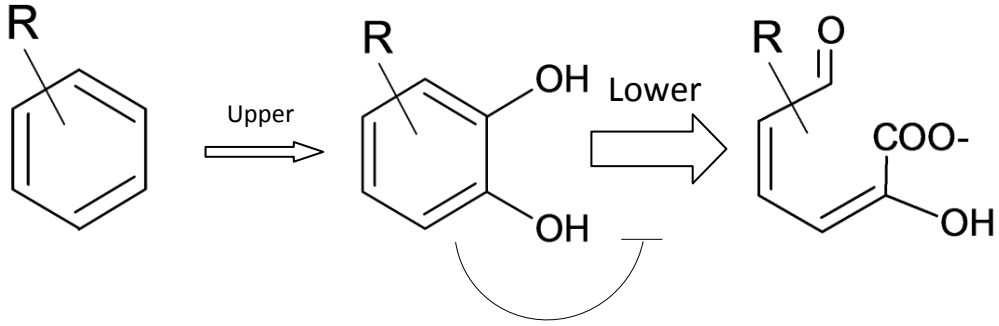


Figure 1.8. Productive scenarios for growth on substrates which produce C23O-inactivating catechols. (a) Although some inactivation still takes place (curved line), selective enhancement of the lower pathway increases catechol turnover and prevents total C23O inactivation. This can be accomplished through changes in gene dosage or mutations which produce more efficient C23Os. **(b)** Some pathways make use of [2Fe-2S] ferredoxins to reactivate C23Os affected by catechol (curved line replaced with circular arrow). This increases catechol turnover and supports growth. **(c)** The "less is more" strategy employed by SF1 is achieved through reduced upper pathway activity which decreases catechol production and subsequent C23O inactivation, thereby allowing for growth.

IV. CONCLUSION

Microorganisms possess a wide range of genetic mechanisms and strategies which allow for adaptation to environmental changes. Current knowledge summarized in this review suggests that the endogenous genetic potential of a bacterium, combined with gene transfer and mutation, allows for the evolution of bacteria capable of growth on a vast array aromatic substrates. As some estimates suggest that we have characterized less than 5 % of microbial diversity (Curtis *et al.*, 2002), it is likely that continued investigations will reveal as yet undiscovered aromatic degradation pathways and provide insight into additional novel mechanisms of adaptation.

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CHAPTER 2

TODF FROM *PSEUDOMONAS PUTIDA* F1 DEGRADES 6-VINYL HODA AND DOES NOT INHIBIT STYRENE METABOLISM

ABSTRACT

Pseudomonas putida F1 cannot grow on styrene despite being able to degrade it through the toluene degradation (*tod*) pathway. Previous work had suggested that this was because TodF, a *meta*-fission product (MFP) hydrolase, was unable to metabolize the styrene MFP, 2-hydroxy-6-vinylhexa-2,4-dienoate (6-vinyl HODA). In this report, we demonstrate via kinetic and growth analyses that the substrate specificity of TodF is not the limiting factor that prevents F1 from growing on styrene. This conclusion is strengthened by preliminary analysis of a styrene-adapted F1 mutant, designated SF1. Taken together, this data suggests that a previously uncharacterized mechanism is responsible for F1's inability to grow on styrene.

INTRODUCTION

Much is known about the mechanisms whereby bacteria degrade aromatic compounds (Diaz, 2004; Khomenkov *et al.*, 2008; van der Meer *et al.*, 1992). Even in model systems, however, we still have much to learn about the determinants of growth substrate specificity. The toluene degradation (*tod*) pathway in *Pseudomonas putida* F1 (Gibson *et al.*, 1968) serves as a prototypical model for aromatic hydrocarbon degradation (Zylstra & Gibson, 1989; Zylstra *et al.*, 1988) (**Figure 2.1**). Multiple substrates are capable of inducing the *tod* operon, however only a subset of these compounds supports growth (Cho *et al.*, 2000; Lacal *et al.*, 2006).

In the *tod* operon, initial dioxygenation of toluene is performed by the multi-component toluene dioxygenase (TDO), a versatile enzyme encoded by *todC1C2BA*

(Jiang *et al.*, 1999; Jiang *et al.*, 1996; Yeh *et al.*, 1977; Zylstra & Gibson, 1989). Following dioxygenation, dehydrogenation of the *cis*-dihydrodiol leads to 3-methylcatechol production. TodE (3-methylcatechol 2,3–dioxygenase) catalyzes the cleavage of this substrate, forming the yellow colored *meta*-fission product (MFP) 2-hydroxy-6-oxo-methylhexa-2,4-dienoate (a.k.a. 6-methyl-HODA) which is then hydrolyzed to acetic acid and 2-hydroxypenta-2,4-dienoate by TodF. After hydrolysis, TodG, TodH, and TodI further degrade the pentadienoate to form pyruvate and acetate (Zylstra *et al.*, 1988). The entire catabolic *todXFC1C2BADEGIH* operon is transcribed from a single promoter designated P_{todX}. Transcriptional activation of this promoter is mediated by TodS and TodT, a constitutively expressed, two-component system located downstream of *todH* (Busch *et al.*, 2007; Lacal *et al.*, 2006; Lacal *et al.*, 2008; Lau *et al.*, 1997; Mosqueda *et al.*, 1999).

Studies of F1 and related *Pseudomonas putida* strains containing the *tod* pathway have implicated TodF (6-methyl HODA hydrolase) as a key determinant of growth substrate specificity, particularly in the metabolism of biphenyl and *n*-alkyl benzenes (Cho *et al.*, 2000; Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ohta *et al.*, 2001; Seah *et al.*, 1998; Seah *et al.*, 2000). In several cases, it has been shown that expression of an alternative *meta*-fission product (MFP) hydrolase such as CmtE from the cumate (cmt) degradation pathway is sufficient to allow these strains to grow on biphenyl (Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ohta *et al.*, 2001). Analysis of available MFP hydrolase crystal structures has revealed a conserved, structural basis for the strict substrate specificity of these enzymes and underscored their important role in aromatic degradation pathways (Fushinobu *et al.*, 2002; Habe *et al.*, 2003; Nandhagopal *et al.*, 2001).

Styrene, despite its ability to induce transcription of the *tod* pathway (Cho *et al.*, 2000; Lacal *et al.*, 2006), cannot be used by *P. putida* F1 for growth. The

observed accumulation of styrene's MFP, 6-vinyl-HODA, has led to the supposition that TodF functions as an enzymatic block in styrene metabolism (Cho *et al.*, 2000). A study reporting the limited substrate range of TodF seemed to corroborate this hypothesis (Seah *et al.*, 1998). There are, however, no published reports on the kinetics of styrene MFP hydrolysis. Here, we present a kinetic analysis of TodF's styrene MFP hydrolase activity and provide evidence that TodF's substrate specificity does not limit styrene metabolism as previously suggested. This conclusion is corroborated by preliminary characterization of SF1, an F1 mutant capable of growth on styrene.

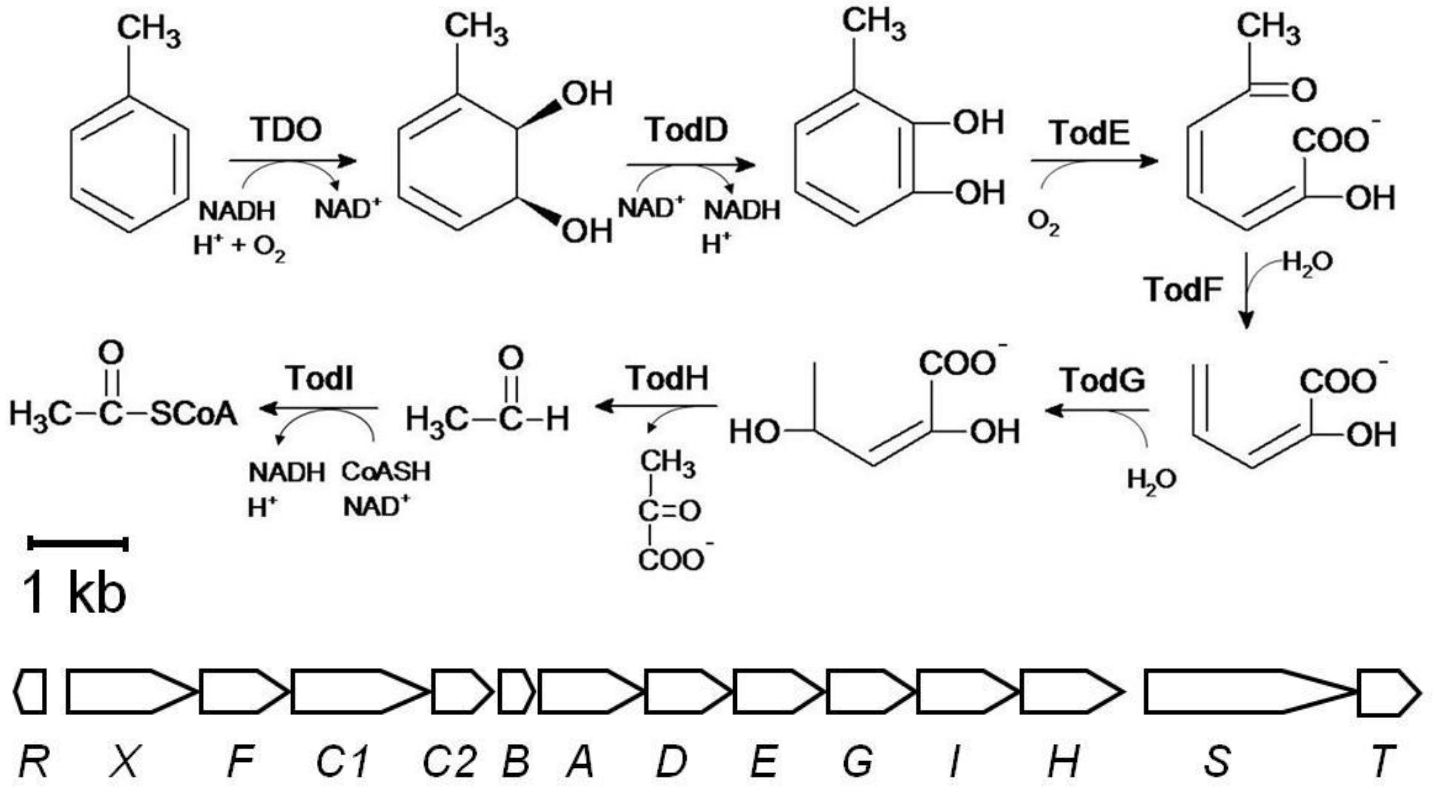


Figure 2.1. **Degradation pathway and genetic organization of the *tod* operon**. The degradation of toluene is pictured. TodF encodes a MFP hydrolase whose limited activity has been proposed to prevent *Pseudomonas putida* F1 from growing on styrene. The *todXFC1C2BADEGIH* operon is transcribed from a single promoter, P_{todX}. Transcriptional activation is mediated by two-component system TodS and TodT, encoded by downstream genes. See text for more detail.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Pseudomonas putida F1 and its plasmid-carrying derivatives were grown at room temperature in LB supplemented with appropriate antibiotics (25 mg l^{-1} and 150 mg l^{-1} for chloramphenicol or ampicillin, respectively) or Minimal Salts Medium (MSM) (Focht, 1994) containing suitable growth substrates. Toluene or styrene were provided in vapor phase for growth on agar plates or at a concentration of 5 mM in liquid media. When specified, acetate was supplied at a concentration of 20 mM. *Escherichia coli* JM109 strains were grown at 37 °C in LB containing 15 mg l^{-1} nalidixic acid. Strains containing pBBR1MCS-5 and derivatives were selected on 25 mg l^{-1} gentamicin. For the isolation of styrene adapted F1 mutants, a dilution series of F1 was plated onto MSM agar and exposed to styrene in vapor phase. After approximately 2 weeks, mutant colonies appeared and were restreaked on to fresh plates. A single mutant capable of robust growth (SF1) was chosen for further study. A complete list of strains and plasmids is presented in **Table 2.1**.

Cloning and DNA manipulation

Primers for genes in the *tod* operon were designed from the genomic sequence of *Pseudomonas putida* F1 (GenBank Accession # NC_009512). A list of primers is presented in **Table 2.2**. PCR was performed using the HotStart Master Mix kit from Qiagen (Valencia, CA) and a PTC-200 DNA Engine thermocycler from MJ Research (Incline Village, NV). PCR fragments were digested with appropriate enzymes and ligated into pBBRMCS-5 (Gm^R) or pRSET-A (Amp^R) using T4 DNA ligase (Promega, Madison, WI). The resultant plasmids were transformed into appropriate hosts by electroporation. Transformants were selected on LB plates containing

antibiotics and screened by PCR. All DNA sequencing was performed by the BioResource Center at Cornell University on an ABI3700 DNA Analyzer.

Preparation of cell-free extracts:

Pseudomonas putida F1 and its derivatives were grown overnight at room temperature on MSM acetate with toluene present in vapor phase as an inducer. JM109 (pTodF) was grown overnight in LB at 37 °C, inoculated 1:100 into fresh media and induced with 1 mM IPTG at an OD_{600} of 0.4. Following induction, cell suspensions were washed and resuspended in sonication buffer (100 mM TRIS-HCl, 10 µM phenylmethylsulphonyl fluoride (PMSF), 1 µM dithiothreitol, pH 8) and lysed through sonication. Supernatants were harvested after centrifugation for 15 minutes (12,000 × g at 4 °C). Protein concentrations of cell-free extracts were determined with a Bradford protein assay (Bio-Rad, Hercules CA) following manufacturer's instructions. Extracts were stored at -20°C in 50% glycerol until use.

Purification of His-tagged TodF

E. coli BL21 (DE3) pLysS (pRSET-A: $TodF_{HIS}$) was grown overnight at 37 °C, inoculated 1:50 into 500 ml fresh SOB media (Hanahan, 1983) and induced with 1 mM IPTG at an OD_{600} of 0.4. After a 16 hour incubation at 37 °C under gentle shaking, cells were harvested through centrifugation (10,000 × g at 4 °C) for 10 minutes and resuspended in Lysis Equilibration Wash (LEW) buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Prior to lysis by sonication, the cell suspension was placed on ice and incubated with lysozyme (0.2 mg ml^{-1}) for 30 minutes with gentle mixing. Crude lysate was collected following centrifugation (12,000 × g at 4 °C) for 15 minutes and added to 0.5 g of Prepease™ (USB Cleveland, OH) Nickel resin. Protein-bound resin was separated through gravity sedimentation and washed

three times with 15 ml of LEW buffer. Three 5 ml aliquots of LEW buffer containing 250 mM imidazole were used to elute $TodF_{His}$.

Biosynthesis of *meta*-fission products

JM109 (pDTG601) (pTodD), a strain containing the entire *tod* operon upper pathway, and S17 (pDmpB), a strain expressing a C23O from the dimethyl phenol pathway (Powlowski & Shingler, 1994), were grown overnight in LB and diluted 1:100 into fresh media of the same type. At mid-exponential phase (OD_{600} = 0.4), IPTG was added to a concentration of 1 mM. Following a 3 hour induction, cultures were washed and resuspended in 30 mM KPO_4 buffer (pH = 7.0). For synthesis of 6-methyl HODA and 6-vinyl HODA, toluene or styrene were added in the vapor phase to a 1:1 co-culture of JM109 (pDTG601) (pTodD) and S17 (pDmpB) until the suspension turned deep yellow indicating the presence of concentrated MFP. Final concentrations of 6-methyl and 6-vinyl HODA were determined spectrophotometrically by measuring absorbance at 389 nm and 425 nm, respectively. A reported extinction coefficient of 11.9 $mM^{-1}cm^{-1}$ was used for 6-methyl HODA and verified experimentally, while an extinction coefficient of 10.7 $mM^{-1}cm^{-1}$ was calculated for 6-vinyl HODA.

Enzymatic Assays

***Meta*-Fission Product (MFP) Degradation:** Degradation of MFPs was quantified by measuring the rate of decrease in absorbance at appropriate wavelengths. For 6-methyl HODA, a wavelength of 389 nm was used while a wavelength of 425 nm was employed for 6-vinyl HODA. Concentrations of MFP were estimated using the appropriate extinction coefficients. Degradation assays were performed at 25 °C

in a 96-well plate format using cell-free extracts in triplicate. Initial degradation rates were measured and standardized to protein content.

Determination of Kinetic Parameters: The kinetic parameters of purified $TodF_{His}$ were determined through measurement of initial degradation rates with varying concentrations of 6-methyl or 6-vinyl HODA. Graphpad Prism 5 ™ (Graphpad Software, Inc. La Jolla, CA) was used to calculate K_M and k_{CAT} based on a least squares analysis of Michaelis-Menten plots.

Quantification of Growth

Growth of F1 and recombinant strains on toluene and styrene was measured using both absorbance at 600 nm and protein content as determined by the Bio-Rad assay.

Table 2.1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
Strains		
P. putida F1	Cm^R, Amp^R; model organism containing the *tod* operon	(Zylstra *et al.*, 1988)
P. putida SF1	Cm^R, Amp^R; Styrene-adapted F1 mutant	This study
E. coli JM109	Nal^R;*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(lac-proAB)	(Yanisch-Perron *et al.*, 1985)
E. coli BL21 (DE3) pLysS	Cm^R, F^- *omp*T *hsd*S_B($r_B^-m_B^-$)*gal* *dcm* (DE3) pLysS	(Miroux & Walker, 1996)
Plasmids		
pBBR1MCS-5	Gm^R; broad host range expression vector	(Kovach *et al.*, 1995)
pRSET A	Amp^R; high level protein expression vector for Ni^{2+} affinity purification	(Kroll *et al.*, 1993)
pDmpB	Derivative of pBBR1MCS-5 containing *dmpB*, DmpB expression vector	This study
pTodF	Derivative of pBBR1MCS-5 containing *todF*, TodF expression vector	This study
pTodD	Derivative of pBBR1MCS-5 containing *todD*, TodD expression vector	This study
pRSET A: $TodF_{HIS}$	Derivative of pRSET A containing *todF* for generation of an N-terminal HIS tagged fusion protein	This study
pDTG601	Derivative of pKK223-3 containing *todC1C2BA*, Used for generation of 3-vinyl and 3-methylcatechol	(Zylstra & Gibson, 1989)

Table 2.2 PCR primers used for DNA amplification and cloning.

All primers were designed and ordered through IDT PrimerQuest (info here). PCR was performed as described in the text. Enzyme restriction sites used for cloning are highlighted.

Name	Sequence	Characteristics
TodF pRSET F	5' – ACA **CTC GAG** ACG AAT GTA AAC GCA GAG ATC GGC – 3'	**XhoI**
TodF pRSET R	5' – ACA **GGT ACC** TTG CCG ATG ACG TGC TTT CAG AAC– 3'	**KpnI**
DmpB Forward	5' – ACA **GGG CCC** CGA CCA CAA CAA CAA GAA CCA GCA – 3'	**ApaI**
DmpB Reverse	5' – ACA **GGA TCC** TTT CAG GTG AGC ACG GTC AGG AAA– 3'	**BamHI**
TodF Forward	5' – ACA **GGG CCC** GTT GCG GCA AAG CCA CAG TCA AAT– 3'	**ApaI**
TodF Reverse	5' – ACA **ATC GAT** TGA CGT GCT TTC AGA ACG GAT GGT– 3'	**ClaI**
TodD Forward	5' – ACA **GGG CCC** CGG CTG GCA GAT CTT TCA AAC AGT– 3'	**ApaI**
TodD Reverse	5' – ACA **GGA TCC** AGC CGA GGT AGC CCA ATC TTT GAA– 3'	**BamHI**

RESULTS

F1 cell-free extract is capable of degrading styrene MFP

To determine if TodF's reported inability to degrade the styrene MFP (Cho *et al.*, 2000) prevented growth on styrene, cell-free extracts of toluene-induced F1were exposed to biologically-synthesized 6-vinyl HODA. Interestingly, F1 degraded 6-vinyl HODA at a considerably higher rate than expected (Cho *et al.*, 2000) (**Figure 2.2a**). Degradation was negligible in an uninduced control, suggesting the involvement of *tod* operon. In order to confirm that TodF was responsible for the observed activity, a *todF* expression vector was constructed (pTodF) and expressed in *E. coli* JM109. Consistent with our initial results, cell-free extracts of induced JM109 (pTodF) rapidly transformed 6-vinyl HODA (**Figure 2.2b**). Both the wild-type and plasmid borne copies of *todF* were sequenced and found to be 100% identical to the previously published sequence (Accession # M64080) (data not shown).

Purified TodF efficiently degrades styrene MFP

To assess TodF activity *in vitro*, we cloned *todF* into pRSET-A and transformed the resultant construct into BL21 (DE3) pLySS, generating an N-terminal HIS-tagged version of the protein. Following purification, an SDS-PAGE gel was run to determine if contamination was present. Non-specific binding was low in all elutions, but elution 2 (**Figure 2.3**) was used to ensure high purity. The molecular weight of the protein matched the expected value for TodF with the addition of the HIS-tag.

$TodF_{HIS}$ was assayed with 6-methyl HODA and 6-vinyl HODA, the ring-fission products of toluene and styrene, respectively. When exposed to both substrates, $TodF_{HIS}$ followed classical Michaelis-Menten kinetics (**Figure 2.3**). Interestingly, the K_M calculated for 6-vinyl HODA (20 μM) was smaller than that for

6-methyl HODA (31 µM), indicating increased enzyme specificity for this substrate. Values for k_{cat}, however, reversed this trend; a value of 32 s^{-1} was calculated for 6-methyl HODA compared to 4.8 s^{-1} for 6-vinyl HODA. k_{cat}/K_M values for both substrates indicated that $TodF_{HIS}$ degraded 6-methyl HODA approximately 4 times more efficiently than 6-vinyl HODA.

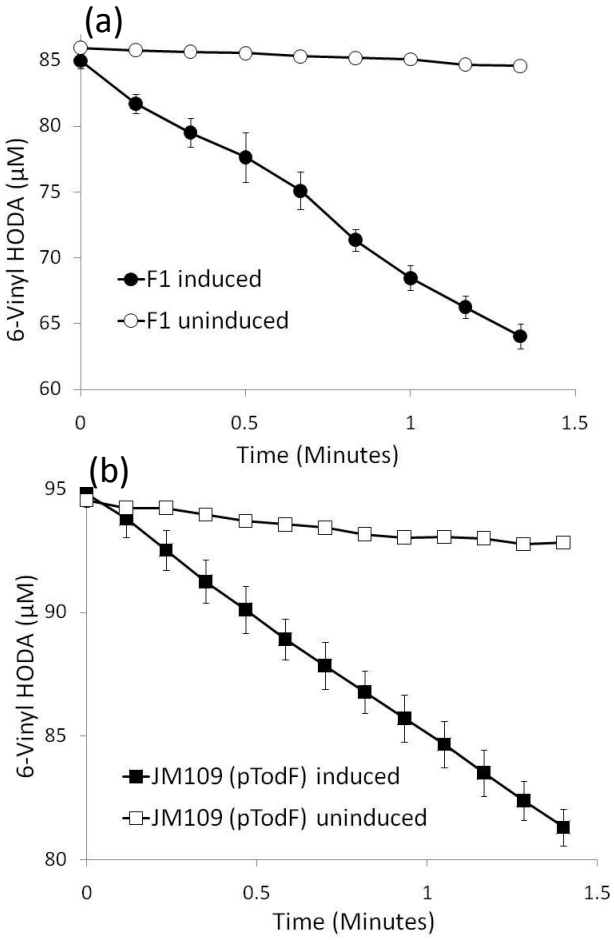
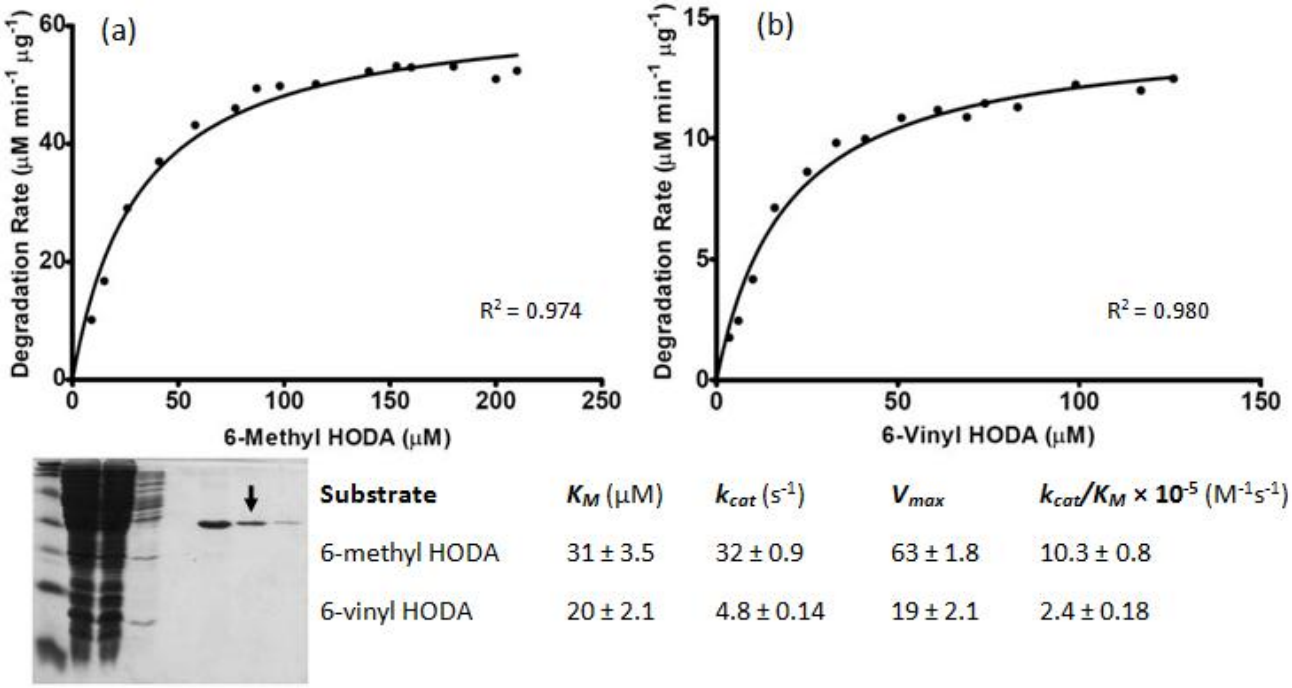


Figure 2.2. Degradation of 6-vinyl HODA by cell free extracts of F1 and JM109 (pTodF). Toluene-grown F1 **(a)** and induced JM109 (pTodF) **(b)** clearly have the ability to degrade the styrene MFP, 6-vinyl HODA. Cell-free extracts were generated as described in methods. Concentrations of 6-vinyl HODA were measured by absorbance at 425 nm. Error bars represent one standard deviation *(n* = 3).



Substrate	K_M (μM)	k_{cat} (s^{-1})	V_{max}	$k_{cat}/K_M \times 10^{-5}$ ($M^{-1}s^{-1}$)
6-methyl HODA	31 ± 3.5	32 ± 0.9	63 ± 1.8	10.3 ± 0.8
6-vinyl HODA	20 ± 2.1	4.8 ± 0.14	19 ± 2.1	2.4 ± 0.18

Figure 2.3. $TodF_{HIS}$ Michaelis-Menten kinetics. $TodF_{HIS}$ is capable of degrading styrene MFP, 6-vinyl HODA, albeit less efficiently than 6-methyl HODA. These curves represent a least-squares fit of the Michaelis-Menten equation to degradation rates of 6-Methyl HODA **(a)** and 6-Vinyl HODA **(b)**. Kinetic parameters for both substrates (± one standard deviation, *n*=3) are listed in the table below the plots. Inset at left: SDS PAGE of $TodF_{HIS}$ purification. Elution 2 is highlighted.

Increased rates of styrene MFP degradation do not confer the ability to grow on styrene

Although F1 is not capable of growth on styrene, styrene-adapted F1 mutants could be isolated on MSM plates following prolonged exposure (1-2 weeks) to styrene in vapor phase (see Methods). Once picked and restreaked, these mutants grew well in both liquid and solid media, reaching stationary phase within 48 hours of styrene exposure. Detailed characterization of one of these mutants, designated SF1, is the focus of Chapter 4.

If limited TodF activity was responsible for precluding growth on styrene, then we hypothesized that a styrene-adapted mutant would be exhibit increased 6-vinyl HODA degradation relative to wild-type. Intriguingly, cell-free extract of toluene-induced SF1 was shown to degrade 6-vinyl HODA at an identical rate to F1 (**Figure 2.4**). Furthermore, DNA sequencing of *todF* in SF1 revealed 100 % identity to WT F1 as did sequencing of the regulatory regions of both the *tod* and *cmt* operons (data not shown). As an additional check, *todF* was sequenced in 8 other styrene-adapted F1 mutants. In every case, *todF* was 100 % identical to WT. These results reinforced the hypothesis that TodF activity was not a block in styrene metabolism. Indeed, increasing TodF activity in F1 above that of SF1 through expression of pTodF (**Figure 2.4**) had no effect on styrene growth (**Figure 2.5**).

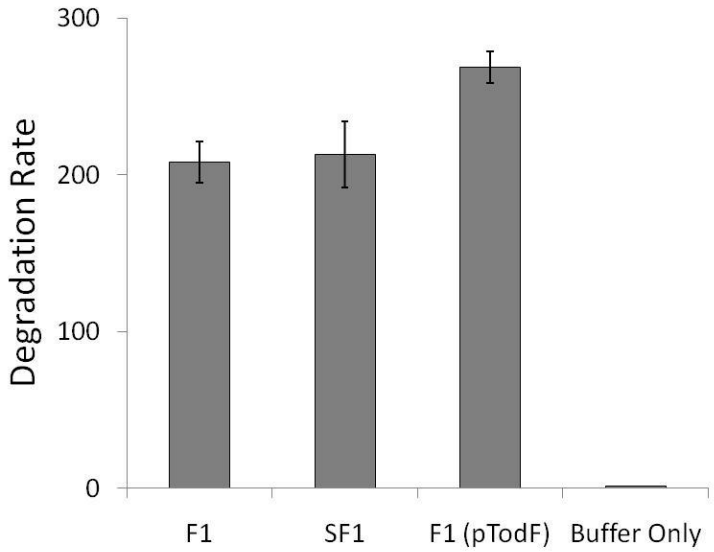


Figure 2.4. 6-vinyl HODA degradation rates of cell-free extracts from F1 and a styrene-adapted mutant (SF1). Toluene-induced F1 and SF1 degrade 6-vinyl HODA at identical rates. Over-expression of TodF in *trans* increases degradation rates in F1. Units for degradation rates are μM min^{-1}ug $protein^{-1}$. Error bars represent one standard deviation (n = 3).

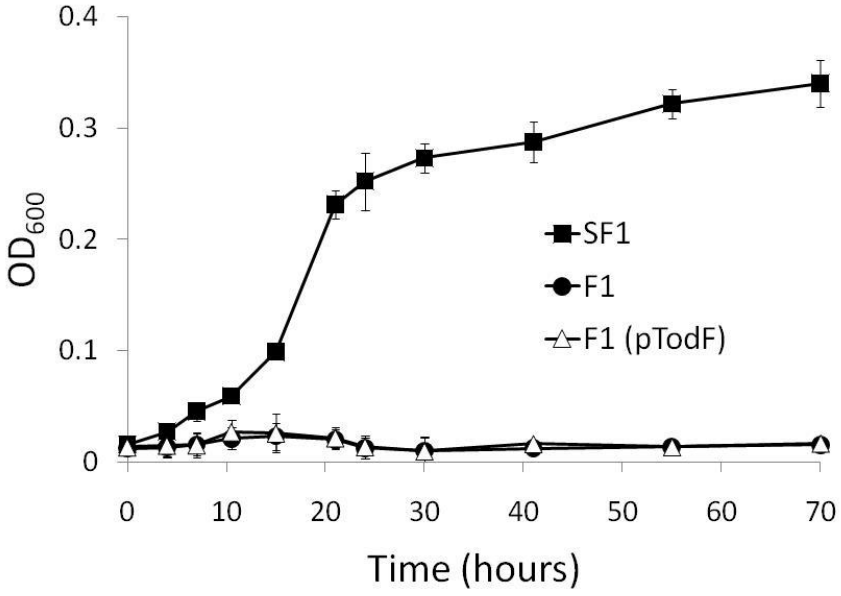


Figure 2.5. Growth of SF1, F1, and F1 (pTodF) on styrene. F1 (pTodF) is unable to grow on styrene despite having increased TodF activity compared to SF1. Error bars represent one standard deviation (n = 3).

DISCUSSION

In this report we have demonstrated that *Pseudomonas putida* F1's inability to grow on styrene is not due to the failure of TodF to metabolize the styrene *meta* fission product (6-vinyl HODA) as has been previously suggested (Cho *et al.*, 2000). We specifically found that purified $TodF_{HIS}$ was able to degrade 6-vinyl HODA at a rate that was slightly lower, but of the same order of magnitude, as 6-methyl HODA. With a K_M of 20 μM TodF's affinity for 6-vinyl HODA was substantially higher than for 6-methyl HODA (31 μM) (**Figure 2.3**). This was not entirely unexpected given that TodF was previously shown to have a lower K_M for the MFP of ethylbenzene, 6-ethyl HODA, than for 6-methyl HODA (Seah *et al.*, 1998). In terms of reaction velocity, however, TodF degraded 6-vinyl HODA considerably slower than 6-methyl HODA, yielding a lower k_{cat} and an approximately 4 fold smaller k_{cat}/K_M. Given that Seah *et al* (1998) reported the highest k_{cat}/K_M for ethylcatechol, this data suggests that the added double bond in 6-vinyl HODA interferes with optimal catalysis. It is unclear if this difference in catalytic efficiency is due to steric interference or the increased chemical stability of 6-vinyl HODA. Regardless, the efficiency of degradation is significant and clearly enough to permit growth. This was definitively demonstrated by SF1, which had identical MFP degradation activity compared to F1 (**Figure 2.4**) but was capable of robust growth on styrene (**Figure 2.5**).

Previous work which implicated the limited activity of TodF as a determinant of growth substrate specificity focused primarily on biphenyl, a substrate that is metabolized to its MFP (6-phenyl-HODA) by the *tod* pathway but does not support growth (Cho *et al.*, 2000; Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ohta *et al.*, 2001) . In several cases, it was demonstrated that mutations which allow for recruitment of an alternative MFP hydrolase, CmtE from the cumate (*cmt*) degradation pathway, permitted growth on biphenyl. Ohta *et al* (2001) worked with *P. putida* CE2010,

demonstrating that a single mutation in the *cmt* promoter impeded repressor (CymR) binding, allowing for constitutive expression of the *cmt* operon and hence growth on biphenyl. Working with F1, Choi *et al.* (2003) demonstrated adaptation to growth on biphenyl required mutations in *cymR* as well as *todS*, allowing for recruitment of CmtE and induction of the *tod* operon, respectively. Additional studies have found similar results, further implicating regulatory mutations in the *cmt* operon (Lee *et al.*, 2006) and the importance of alternative MFP hydrolases (Furukawa *et al.*, 1993). Given that SF1 had identical 6-vinyl HODA degradation activity compared to F1, it is unlikely that recruitment of an alternative MFP hydrolase played a role in SF1's ability to grow on styrene. Indeed, DNA sequencing of *cmt* regulatory regions in SF1 revealed 100 % identity to wild-type. This data clearly shows that SF1 possesses unique adaption(s) which allow it to grow on styrene.

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CHAPTER 3

SUICIDE CATECHOL-2,3-DIOXYGENASE INACTIVATION LIMITS SUBSTRATE RANGE IN PSEUDOMONAS PUTIDA F1 AND PREVENTS GROWTH ON STYRENE

ABSTRACT

We previously demonstrated that *Pseudomonas putida* F1's inability to grow on styrene was not due to limited TodF activity. In this work, we show that suicide inactivation of TodE, the catechol-2,3-dioxygenase (C23O) of the *tod* operon, is responsible for preventing F1 from growing on styrene. We found that the metabolite 3-vinylcatechol accumulated during styrene metabolism and that micro molar concentrations of this intermediate inactivated TodE. Analysis of cells growing on styrene suggested that inactivation of TodE and the subsequent accumulation of 3-vinylcatechol resulted in toxicity and cell death. Intriguingly, we found that simply over-expressing TodE on a plasmid (pTodE) was all that was necessary to allow F1 to grow on styrene. Similar results were also obtained by expressing a related C23O, DmpB from *Pseudomonas* sp. CF600, in tandem with its plant-like ferredoxin, DmpQ (pDmpQB). Further analysis revealed that F1 (pDmpQB) and F1 (pTodE)'s ability to grow on styrene correlated with increased C23O activity as well as the enzymes' resistance to vinylcatechol-mediated inactivation. Although TodE inactivation by 3-halocatechols has been studied before, to our knowledge, this is the first published report demonstrating inactivation by a 3-alkylcatechol. Given the ubiquity of catechol intermediates in aromatic hydrocarbon metabolism, our results further demonstrate the importance of C23O inactivation as a determinant of growth substrate specificity.

INTRODUCTION

Ring cleavage is perhaps the most critical and highly-conserved step in aromatic degradation pathways (van der Meer *et al.*, 1992). Mechanistically, ring fission is divided into two categories: In *ortho*- cleavage, dioxygenation takes place at the 1,2- position of the catechol (within the hydroxyl groups), while in *meta*-cleavage fission occurs at the 2,3- position (outside the hydroxyls) (Harayama & Rekik, 1989). *Ortho*- and *meta*- cleavage are catalyzed by intradiol and extradiol dioxygenases, which use Fe (III) and Fe (II) at the active site to catalyze ring cleavage, respectively (Harayama *et al.*, 1992). Although these differences between the dioxygenases seem trivial, the enzymes have vastly different structures and dissimilar catalytic mechanisms (Brivio *et al.*, 2009). In general, extradiol dioxygenases (catechol-2,3-dioxgenases, C23Os) demonstrate superior versatility and act on a wider range of substrates (Vaillancourt *et al.*, 2006).

The mechanism by which C23Os catalyze ring cleavage involves a non-heme Fe (II) situated in the active site, usually surrounded by a hydrophobic pocket. This active-site ferrous iron is coordinated by a conserved structural motif consisting of two histidines and one glutamate (Vaillancourt *et al.*, 2006). Given the importance of the ring-cleavage step, the substrate specificity of C23Os is often the key determinant of pathway specificity and consequently an organism's capacity for growth.

During the course of catalysis, C23Os may become inactivated by their substrates in a process known suicide inhibition (Bartels *et al.*, 1984; Klecka & Gibson, 1981). Suicide inhibition appears to involve oxidation of active site Fe (II) to Fe (III) (Cerdan *et al.*, 1994; Vaillancourt *et al.*, 2002) and has been demonstrated most often with chlorocatechols and *para*- substituted alkyl catechols (Vaillancourt *et al.*, 2006). In aromatic degradation pathways, mechanism-based C23O inactivation often results in catechol accumulation and has been shown to limit the substrate range

of the TOL, biphenyl (bph), and toluene degradation (*tod*) pathways (Cerdan *et al.*, 1995; Cerdan *et al.*, 1994; Klecka & Gibson, 1981; Ramos *et al.*, 1987; Rojo *et al.*, 1987; Vaillancourt *et al.*, 2002; Ward *et al.*, 2004).

In vitro, enzymatic inactivation of C23Os can be reversed by treatment with reducing agents or incubation under anaerobic conditions with Fe (II) (Vaillancourt *et al.*, 2006). *In vivo,* some organisms appear to utilize separate proteins, [2Fe-2S] plant-like ferredoxins, to reactivate oxidized C23Os and restore proper function. This has been most clearly demonstrated in the case of XylT, a novel plant-like [2Fe-2S] ferredoxin in the TOL pathway shown to reactivate XylE, a C23O homologous to TodE (Hugo *et al.*, 1998; Hugo *et al.*, 2000; Polissi & Harayama, 1993). Similar enzymes are encoded in other aromatic degradation operons including DmpQ, a XylT-like ferredoxin in the *dmp* operon encoding the metabolism of phenols in *Pseudomonas* sp. CF600 (Powlowski & Shingler, 1994). Like XylT, DmpQ is capable of reactivating XylE, suggesting a conserved mechanism of action for [2Fe-2S] ferredoxins (Hugo *et al.*, 2000). By serving as a natural repair system in aromatic degradation pathways, it is likely that [2Fe-2S] ferredoxins play key roles in augmenting the range of growth substrates in numerous organisms.

Pseudomonas putida F1 is unable to grow on styrene despite metabolizing it through the toluene degradation (*tod*) operon. Although earlier studies suggested this was due to the limited activity of TodF towards 6-vinyl HODA (Cho *et al.*, 2000), our previous work (Chapter 2) indicated that TodF was capable of relatively efficient metabolism of the styrene MFP. In this study, we demonstrate that suicide inhibition of TodE, the C23O of the toluene degradation (*tod*) operon, is the limiting metabolic factor which prevents *Pseudomonas putida* F1 from growing on styrene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Pseudomonas putida F1 and derivative plasmid-carrying strains were grown under gentle shaking at room temperature in Minimal Salts Medium (MSM) (Focht, 1994) containing appropriate substrates. Toluene or styrene was provided as vapor for growth on agar plates or at a concentration of 5 mM in liquid media. When specified, acetate was supplied at a concentration of 20 mM. *Escherichia coli* S17-1 λpir (Delorenzo & Timmis, 1994) strains were grown at 37 °C in LB containing 50 μg ml^{-1} streptomycin. Strains containing pBBR1MCS-5 were selected on 25 μg ml^{-1} gentamicin. A complete list of strains and plasmids is presented in **Table 3.1**.

Cloning and DNA manipulation.

Primers for genes in the *tod* and *dmp* operons were designed based on published sequences from *Pseudomonas putida* F1 (GenBank Accession # NC_009512) and *Pseudomonas* sp. CF600 (GenBank Accession #X52805), respectively. Primer sequences are provided in **Table 3.2**. PCR was performed with the HotStart Master Mix kit from Qiagen (Valencia, CA) and a PTC-200 DNA Engine thermocycler from MJ Research (Incline Village, NV). For cloning, PCR fragments were digested with restriction enzymes and ligated into pBBRMCS-5 (Gm^R) using T4 DNA ligase (Promega, Madison, WI). Electroporation was used for host transformation. Transformants were selected on LB agar containing suitable antibiotics and screened by PCR. All DNA sequencing was performed by the BioResource Center at Cornell University using an ABI3700 DNA Analyzer.

GC-MS detection of 3-vinylcatechol

F1 was grown for 48 h on MSM styrene + acetate. After removal of cells via centrifugation (10,000 × g at 4°C for 10 minutes), intermediates in the supernatant were derivatized with 1.5% K_2CO_3 and 0.5% acetic anhydride (Baker *et al.*, 1994). Following extraction with chloroform, samples were analyzed on a Hewlett Packard 6890 GC coupled to a HP 5973 MS detector. An HP-5MS column (29.2 mm × 0.25 mm × 0.25 μm) was used with a temperature range of 60 °C to 300 °C at a ramp of 30 °C/min.

Biosynthesis of 3-vinylcatechol

JM109 (pDTG601) (pTodD) was grown overnight in LB and diluted 1:100 into fresh media of the same type. At mid-exponential phase (OD_{600} = 0.4), IPTG was added to a concentration of 1 mM. Following a 3 hour induction, cultures were washed and resuspended in 30 mM KPO_4 buffer (PB) (pH = 7.0). For synthesis of 3-vinylcatechol, styrene was added to a concentration of 1 mM and the cultures were subsequently incubated for 8 hours at 37 °C under gentle mixing. The supernatant was then harvested following centrifugation (10,000 × g at 4°C for 15 minutes), filter sterilized, and kept on ice prior to use. GC-MS analysis of the supernatant revealed a single robust peak with a mass spectrum consistent with 3-vinylcatechol (data not shown).

Resting cell assays

For resting cell enzymatic assays, F1 and relevant strains were grown to late exponential phase (OD_{600} = 0.8) at room temperature in MSM acetate and inoculated 1:100 into fresh MSM media containing acetate + toluene. Upon reaching late

exponential phase, cells were harvested by centrifugation (10,000 x g for 10 min at 4 °C), washed twice in 30 mM PB, and resuspended in an equal volume of sterile buffer.

Measurement of C23O activity. C23O activity was measured as the rate of increase in A_{375} following the addition of 2 mM catechol as described previously (Salat-Trepat & Evans, 1971). Briefly, 200 µL volumes of resting cells in PB were assayed in triplicate using a Bio-Tek (Winooski, VT) Synergy^{TM} HT-I microplate reader. Following the addition of catechol, initial rates of *meta*-cleavage were calculated and correlated with protein content as determined with a Bradford assay (Bio-Rad, Hercules CA) following manufacturer's instructions. Concentrations of catechol *meta*-fission product were calculated using a reported extinction coefficient of 40 $mM^{-1}cm^{-1}$ (Seah *et al.*, 1998).

C23O inactivation assays. To quantify C23O inactivation following substrate cleavage, cultures were exposed to 2 mM of the various substrates. For H_2O_2 – catalyzed C23O inactivation (Hugo *et al.*, 1998), various concentrations were used to generate a dose-response curve. For 3-vinylcatechol exposures, cultures were resuspended in filtered PB containing 50 µM of substrate. At set time points, 1 mL aliquots were taken, centrifuged at 16,000 × g for 1 minute, and resuspended in an equal volume of PB. C23O activity was measured as described above.

Table 3.1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
Strains		
P. putida F1	Cm^R, Amp^R; model organism containing the *tod* operon	(Zylstra *et al.*, 1988)
E. coli S17-1 λpir	λ*pir*; *hsdR pro recA*; RP4 2-Tc : : Mu-Km : : Tn7, pro, res^-, mod^+, Str^R, Trm^R	(Delorenzo & Timmis, 1994)
Plasmids		
pBBR1MCS-5	Gm^R; broad host range expression vector	(Kovach *et al.*, 1995)
pDmpB	Derivative of pBBR1MCS-5 containing *dmpB*, DmpB expression vector	This study
pDmpQ	Derivative of pBBR1MCS-5 containing *dmpQ*, DmpQ expression vector	This study
pDmpQB	Derivative of pBBR1MCS-5 containing *dmpQB*, DmpQB expression vector	This study
pTodE	Derivative of pBBR1MCS-5 containing *todE*, TodE expression vector	This study
pDTG601	Derivative of pKK223-3 containing *todC1C2BA*, Used for generation of 3-vinyl and 3-methylcatechol	(Zylstra & Gibson, 1989)
pTodD	Derivative of pBBR1MCS-5 containing *todD*, TodD expression vector	This study

Table 3.2. PCR primers used in this study for DNA amplification and cloning. All primers were designed and ordered through IDT PrimerQuest™. PCR was performed as described in the text. Enzyme restriction sites used for cloning are highlighted.

Name	Sequence	Characteristics
TodE Forward	5' – ACA **GGG CCC** ACA AGC ACT TCG GTT GAG ACA GGA – 3'	**ApaI**
TodE Reverse	5' – ACA **GGA TCC** TGG CAT ATG GGC ATC CAG GTA GAA – 3'	**BamHI**
DmpQ Forward	5' – ACA **GGG CCC** AAG CGC ATC TGA GGT GAA CCA TGA – 3'	**ApaI**
DmpQ Reverse	5' – ACA **GGA TCC** TTC ATG ACG ACA CCT CTT GCT GGT – 3'	**BamHI**
DmpB Forward	5' – ACA **GGG CCC** CGA CCA CAA CAA CAA GAA CCA GCA – 3'	**ApaI**
DmpB Reverse	5' – ACA **GGA TCC** TTT CAG GTG AGC ACG GTC AGG AAA– 3'	**BamHI**
TodF Forward	5' – ACA **GGG CCC** GTT GCG GCA AAG CCA CAG TCA AAT– 3'	**ApaI**
TodF Reverse	5' – ACA **ATC GAT** TGA CGT GCT TTC AGA ACG GAT GGT– 3'	**ClaI**
TodD Forward	5' – ACA **GGG CCC** CGG CTG GCA GAT CTT TCA AAC AGT– 3'	**ApaI**
TodD Reverse	5' – ACA **GGA TCC** AGC CGA GGT AGC CCA ATC TTT GAA– 3'	**BamHI**

RESULTS

F1 exposed to styrene accumulates 3-vinylcatechol.

When resting F1 cells were exposed to styrene vapors, a yellow-colored compound with an absorbance spectrum consistent with 6-vinyl HODA was observed in the culture medium (data not shown). The appearance of this *meta*-fission product was expected based on a previous report (Cho *et al.*, 2000). Its accumulation was transient, however, and after approximately 48 hours the culture had turned a deep, opaque brown (**Figure 3.1**, inset). In resting cells exposed to toluene, no such color was evident. Given that catechol polymerization products are typically brown or black in color, it was hypothesized that 3-vinylcatechol had accumulated in the culture media and subsequently polymerized. GC/MS Analysis of chloroform extracts of the acetylated supernatant revealed a single, large peak with a mass spectrum consistent with acetylated 3-vinylcatechol (**Figure 3.1**). No catecholic intermediates were detected in cells exposed to toluene (data not shown). The accumulation of 3-vinylcatechol suggested that TodE, the C23O responsible for cleaving this intermediate, might be inactivated during styrene metabolism.

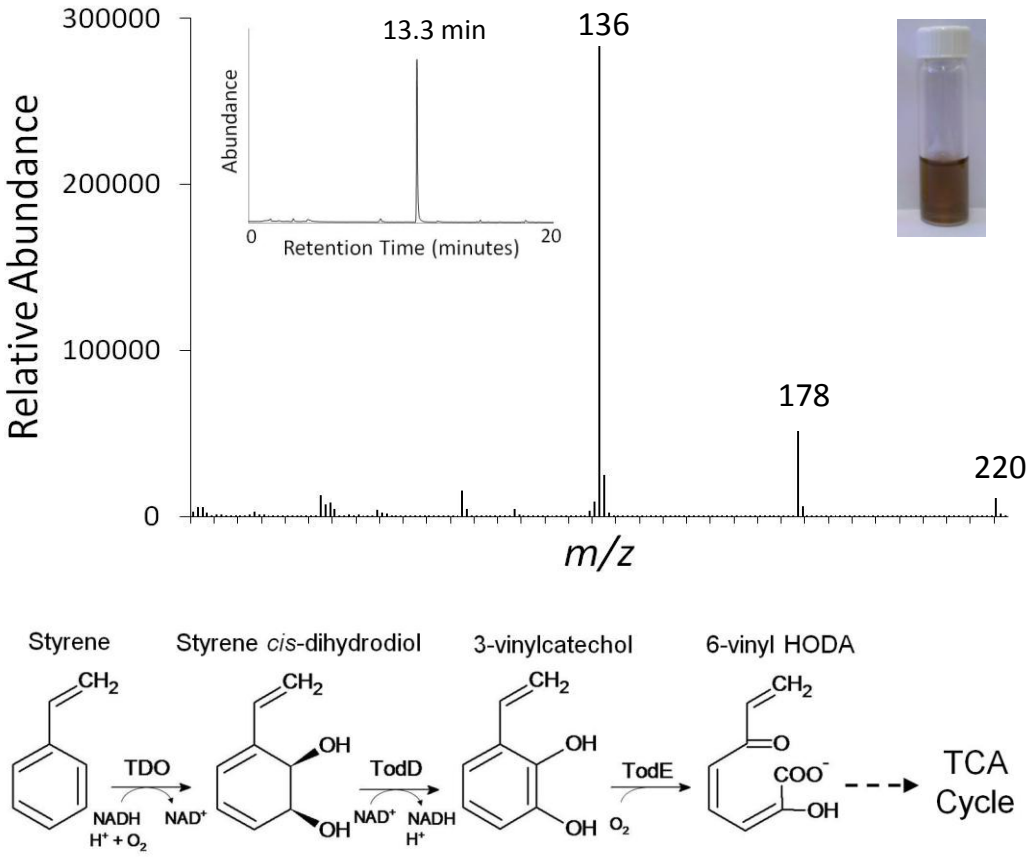

Figure 3.1. Mass spectrum of acetylated 3-vinylcatechol. When toluene-grown F1 cultures were exposed to styrene they accumulated high levels of of a brown product putatively identified as polymerized 3-vinylcatechol. Prior to the appearance of opaque brown polymerization product (inset at right), 4 ml of the culture was acetylated and extracted in 1 ml chloroform (see methods). A single large peak (see inset at left) was detected with a mass spectrum consistent with acetylated 3-vinylcatechol. Pictured below is the partial pathway of styrene degradation in F1. The accumulation of 3-vinylcatechol suggested impaired C23O (TodE) function.

C23O activity is abolished in *Pseudomonas putida* F1 exposed to styrene and 3-vinylcatechol.

Based on the accumulation of 3-vinylcatechol, it was hypothesized that styrene metabolism resulted in the abrogation of C23O activity in growing F1 cells. To test this hypothesis, resting cultures of toluene-induced F1 were exposed to styrene and its intermediate 3-vinylcatechol and assayed for C23O activity. Interestingly, within 1 hour of exposure to styrene, C23O activity was reduced 7-fold in toluene-induced cells (**Figure 3.2a**). Exposure of similarly induced cells to toluene caused no such inactivation. 3-Vinylcatechol, the substrate directly cleaved by TodE (see **Figure 3.1**), was an even more potent inhibitor and appeared to abolish C23O activity completely within 2 hours (**Figure 3.2a**). Inactivation appeared to be concomitant with the appearance of 6-vinyl HODA (data not shown). 3-Methylcatechol generated from toluene served as a control and had no effect on C23O activity.

To determine if 3-vinylcatechol was the actual inhibitor of C23O activity and if metabolism of styrene to 3-vinylcatechol was required for C23O inactivation, a *todE* expression vector (pTodE) was constructed and expressed in *E. coli* S17. The resultant strain was then exposed to either styrene or 3-vinylcatechol. As expected, C23O activity in S17 (pTodE) was abrogated by exposure to 3-vinylcatechol, but unaffected by styrene (**Figure 3.2b**). The relevant controls of toluene and 3-methylcatechol had no effect on C23O activity.

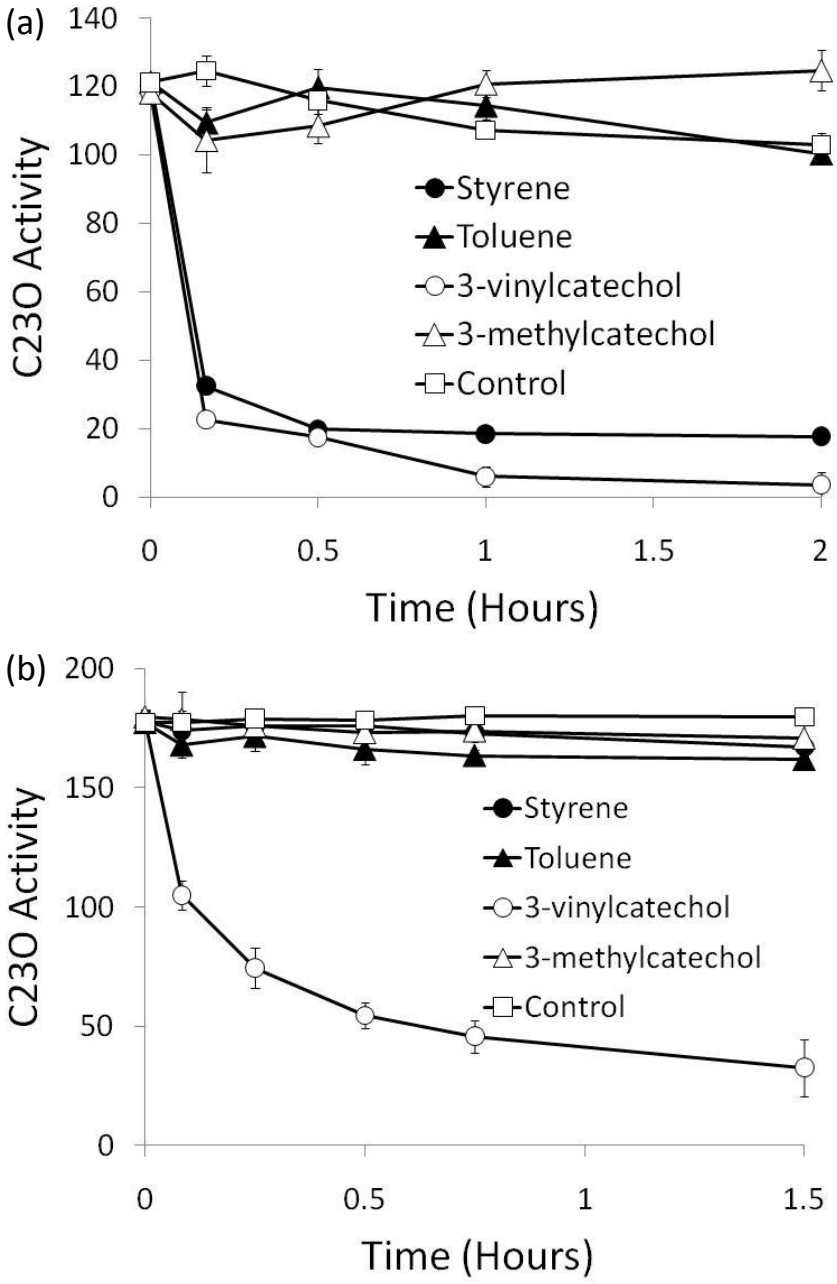

Figure 3.2. C23O inactivation following exposure to styrene and 3-vinylcatechol. In resting cultures of *P. putida* F1, exposure to either styrene or 3-vinylcatechol resulted in the significant reduction of C23O activity. Neither toluene nor its metabolite 3-methylcatechol had any effect **(a)**. In *E. coli* S17 (pTodE), only 3-vinylcatechol caused C23O inactivation **(b)**. In both panels, C23O activity was measured following the addition of 2 mM catechol to washed cells (see methods). Units of C23O activity are $\mu M\ min^{-1}\ mg\ protein^{-1}$. Error bars represent one standard deviation ($n = 3$).

Expression of DmpQB and TodE in *trans* permits growth on styrene.

Given that C23O inactivation was implicated as being responsible for F1's inability to grow on styrene, it was hypothesized that increasing the levels of C23O activity through the expression of plasmid-borne C23O genes and/or a plant-like ferredoxin would permit F1 to use styrene as a growth substrate.

F1 has no plant-like ferredoxin of its own, so an expression vector encoding the ferredoxin DmpQ from *Pseudomonas* CF600 (Powlowski & Shingler, 1994), designated pDmpQ, was constructed and expressed in F1. pDmpQB, which expressed DmpQ together with its endogenous C23O partner DmpB, was also created and heterologously expressed in F1. This way, DmpQ would rescue the C23O activity of DmpB even if, by chance, it did not rescue that of TodE. Expression of pDmpB and pDmpQB in *E. coli* proved that both gene products were functional, with DmpQ conferring resistance to H_2O_2-catalyzed C23O inactivation (**Figure 3.3**). An F1 strain carrying the TodE expression vector pTodE was also was also analyzed to determine if simply increasing basal levels of F1's native C23O enzyme could yield similar results.

Analysis of CFUs from cultures grown in MSM with styrene yielded surprising results (**Figure 3.4**). Intriguingly, all F1 strains were capable of modest initial growth on styrene in the first 28 hours. By 60 hours, however, it was apparent that F1 WT had experienced cell-death with a striking reduction in CFUs compared to time 0. At 60 hours, F1 (pDmpQ) and F1 (pDmpB) CFUs were an order of magnitude larger than F1, but not significantly different than time 0 ($P>0.05$). Although F1 (pDmpQ) was unable to grow on styrene, the increased CFU count at 60 h compared to F1 suggested a modest protective effect. Consistent with this observation, an analysis of F1 (pDmpQ)'s C23O activity following styrene exposure demonstrated that expression of DmpQ conferred slightly increased resistance to C23O inactivation compared to F1 (**Figure 3.5**). Brown catechol polymerization products were evident

in all strains which experienced a reduction in CFUs after 28 hours and indicated accumulation of 3-vinylcatechol.

Neither F1 (pDmpQB) nor F1 (pTodE) accumulated 3-vinylcatechol and both strains were able to grow on styrene. F1 (pTodE) grew noticeably slower than F1 (pDmpQB), although CFUs from both strains were nearly 3 orders of magnitude higher than F1 after 60 hours. Importantly, as additional evidence that TodF activity was not limiting growth on styrene (see Chapter 2), the rates of 6-vinyl HODA degradation in F1 (pDmpQB) and F1 (pTodE) were found to be identical to wild type (data not shown). Sequencing also revealed that both strains harbored wild type *todF* alleles.

To gain further insight into the relationship between C23O inactivation and growth, resting cultures of *E. coli* S17 (pDmpQB), S17 (pDmpB), and S17 (pTodE) were exposed to 3-vinylcatechol and analyzed for C23O activity. The initial rate of 3-vinylcatechol cleavage was highest in S17 (pDmpQB), with a C23O activity of 900 μM mg protein^{-1}min^{-1}, approximately 2.5 and 2 fold higher than S17 (pTodE) and S17 (pDmpB), respectively (**Figure 3.6a**). Although initial rates of 3-vinylcatechol cleavage were comparable between DmpB and TodE, inactivation behavior was clearly distinct: exposure to 100 μM 3-vinylcatechol completely abolished C23O activity in S17 (pDmpB), but S17 (pTodE) retained residual activity even after exposure to 200 μM of substrate (**Figure 3.6b**). Intriguingly, S17 (pTodE) appeared to be even more resistant to inactivation than S17 (pDmpQB). A time course of 3-vinylcatechol cleavage by the C23O-bearing S17 strains is presented in **Figure 3.7**.

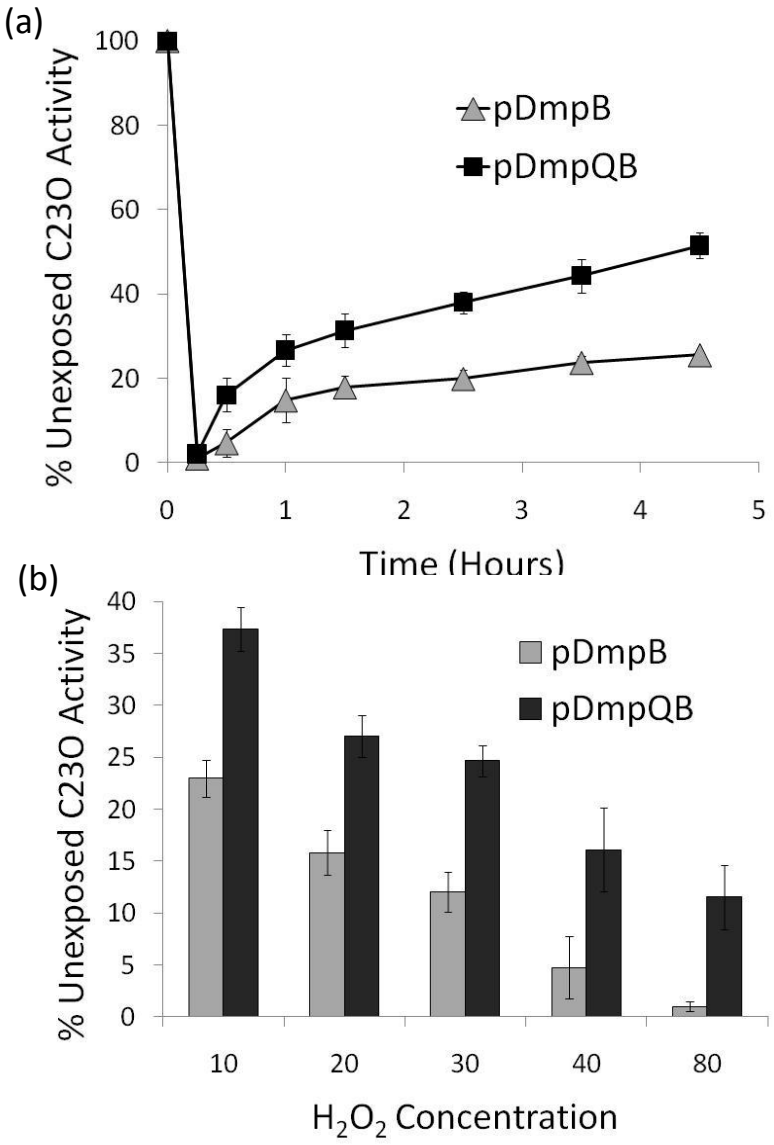

Figure 3.3. DmpQ aids in the reactivation of DmpB. S17 (pDmpQB) recovers C23O activity more rapidly and to a greater extent than S17 (pDmpB) following total inactivation by 40 mM H_2O_2 **(a)**. S17 (pDmpQB) is more resistant to inactivation than S17 (pDmpB) following a 30 minute exposure to all tested concentrations of H_2O_2 **(b)**. In both plots, C23O activity is expressed relative to an unexposed control. Error bars represent one standard deviation (n = 3).

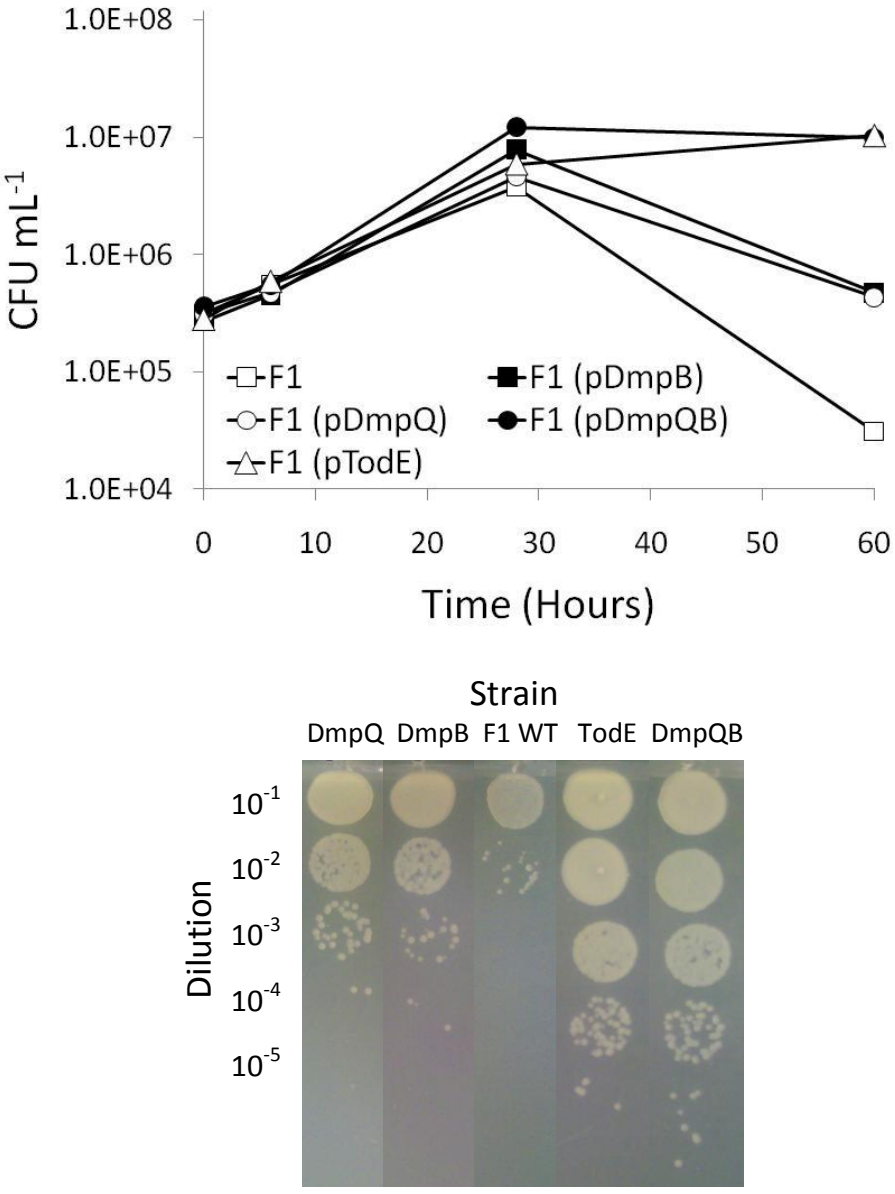

Figure 3.4. Growth of F1 and recombinant strains on styrene. Although all strains initially grew on styrene, only those strains over expressing C23Os and/or a ferredoxin were able to avoid the toxicity and cell death associated with 3-vinylcatechol accumulation. A representative dilution series for each strain after 60 hours of growth is shown. Error bars represent one standard deviation (n = 3).

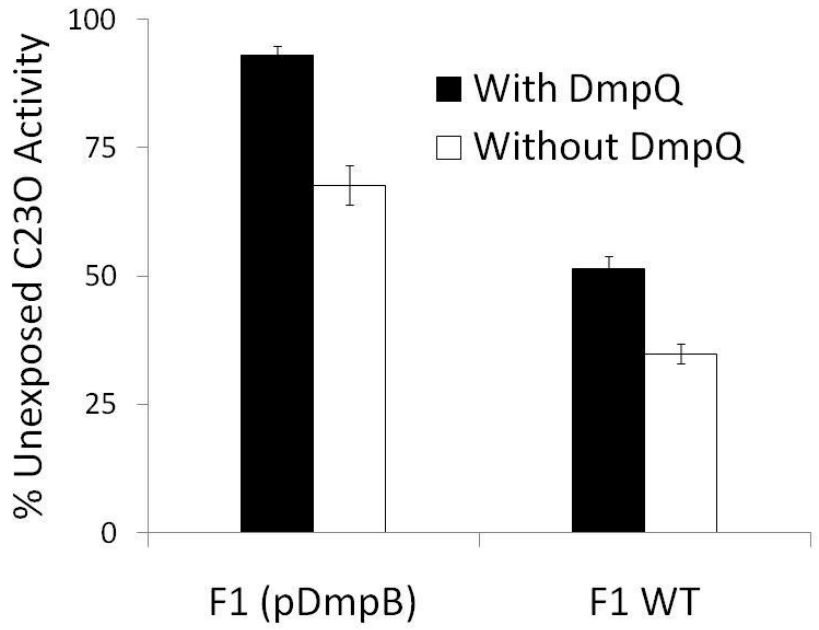

Figure 3.5. The effect of DmpQ on C23O inactivation following styrene exposure. Following exposure to styrene (2 mM) for 3 hours, C23O activity was measured using catechol both F1 (pDmpB) and F1 WT with and without co-expression of DmpQ. In both cases, DmpQ conferred increased resistance to C23O inactivation. Unexposed F1 and F1 (pDmpQ) controls had C23O activities of 160 μM $min^{-1}mgprotein^{-1}$, while F1 (pDmpB) and F1 (pDmpQB) controls had C23O activities of 1600 μM $min^{-1}mgprotein^{-1}$ and 4200 μM $min^{-1}mgprotein^{-1}$, respectively. Error bars represent one standard deviation (n = 3).

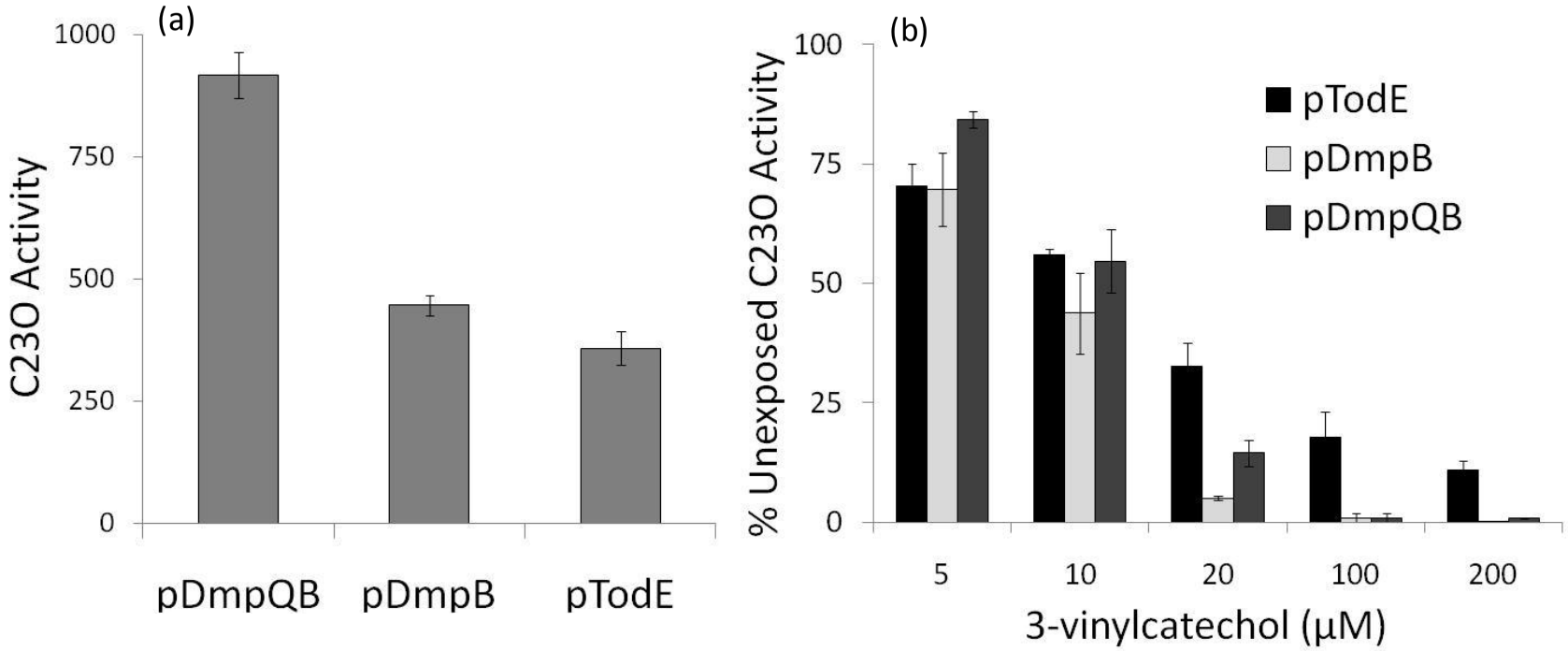

Figure 3.6. C23O activity and inactivation behavior of *E. coli* strains expressing relevant C23Os. S17 (pDmpQB) had the highest initial C23O activity following exposure to 3-vinylcatechol, while S17 (pDmpB) and S17 (pTodE) yielded similar activities **(a)**. Despite having the lowest initial activity, TodE was more resistant than both DmpB and DmpQB to 3-vinylcatechol-mediated inactivation **(b)**. Units of C23O activity are µM min^{-1} mg $protein^{-1}$. Measurements were taken following a 30 minute exposure to 3-vinylcatechol. Error bars represent one standard deviation (n = 3).

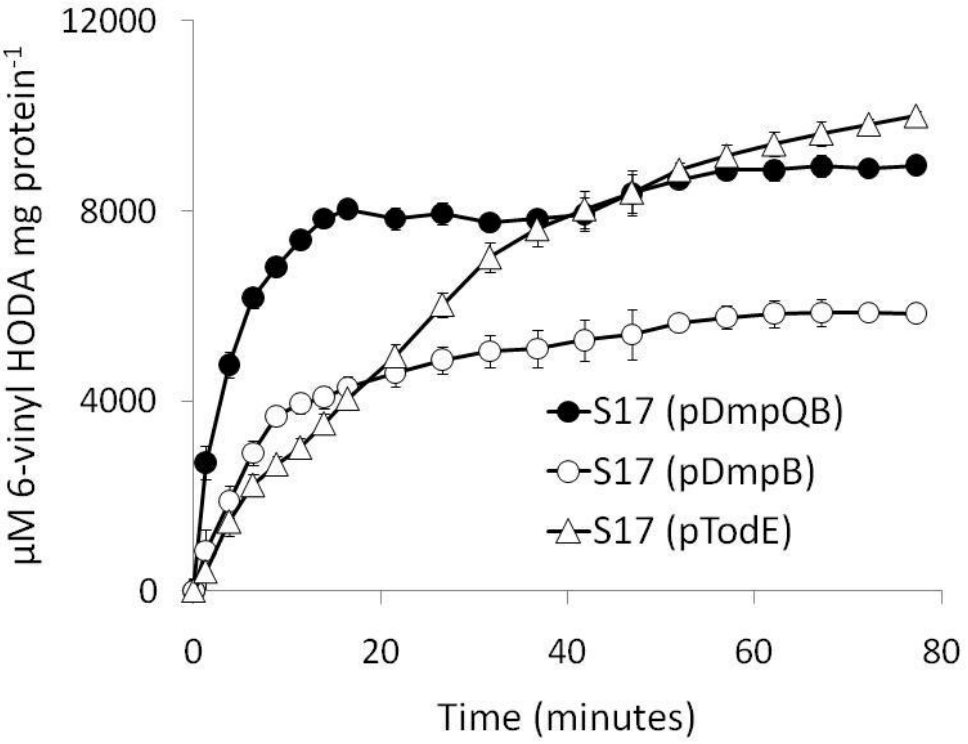

Figure 3.7. Cleavage of 3-vinylcatechol by *E. coli* strains harboring C23Os. Despite initially cleaving 3-vinylcatechol at the slowest rate, S17 (pTodE) was eventually able to transform the largest amount of substrate to 6-vinyl HODA. S17 (pDmpQB) rapidly degraded 3-vinylcatechol, but was inactivated prior to total transformation of the substrate. Error bars represent one standard deviation (n = 3).

DISCUSSION

In this report we have demonstrated that *Pseudomonas putida* F1's inability to grow on styrene is due to suicide inactivation of TodE during cleavage of 3-vinylcatechol. This inactivation prevents the conversion of 3-vinylcatechol to intermediates that support growth, but more importantly, causes 3-vinylcatechol to accumulate resulting in cell death. We have shown that increasing C23O expression prevents 3-vinylcatechol accumulation and permits F1 to grow on styrene. These findings corroborate those from Chapter 2 and suggest an expanded role for C23O inactivation as a determinant of growth substrate specificity.

Previous studies have demonstrated multiple means for expanding the substrate range of aromatic degradation pathways including mutations in regulation, recruitment of alternative enzymes, and the expression of "repair" enzymes such as [2Fe-2S] ferredoxins (Cerdan *et al.*, 1994; Cho *et al.*, 2000; Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ramos *et al.*, 1987; Rojo *et al.*, 1987). With respect to the *tod* operon, work has focused on TodF and the expansion of substrate range through the recruitment of alternative MFP hydrolases, BphD (Furukawa *et al.*, 1993) and CmtE (Choi *et al.*, 2003).

In Chapter 2, we specifically found that purified $TodF_{HIS}$ was able to degrade 6-vinyl HODA at a rate that was slightly lower, but of the same order of magnitude, as 6-methyl HODA. The observation that TodF degrades 6-vinyl HODA, coupled with our finding that F1 and derivatives all initially grew on styrene (**Figure 3.5**), suggest that enough styrene was metabolized to citric acid cycle intermediates to support growth. This conclusion is also supported by our finding that F1 DmpQB and F1 TodE, which grew well on styrene, still harbored wild type *todF* alleles and activity. Taken together, these findings are strong evidence that TodF activity is not the factor preventing growth on styrene. Rather, the ability of the F1 derivatives over-

expressing C23Os to grow on styrene – together with the effect of 3-vinylcatechol on TodE activity – make it clear that inactivation of TodE is responsible for the accumulation of 3-vinylcatechol, 3-vinylcatechol toxicity, and F1's lack of net growth on styrene after 60 hours.

Interestingly, simply increasing the basal 3-vinylcatechol C23O activity in F1 2 fold through expression of DmpB was not enough to permit F1 (pDmpB) to grow on styrene, even though a similar level of C23O activity enhancement permitted growth of F1 (pTodE) (**Figure** 3.**5**). Further analysis suggested that this was because DmpB was actually more sensitive to the deactivating affects of 3-vinylcatechol than TodE (**Figure 3.6b**) even though they had similar initial activity (**Figure 3.6a**). The similarity in initial activity levels is also good evidence that apparent differences in resistance to C23O inactivation were not just due to differences in heterologous protein expression.

When co-expressed with its endogenous ferredoxin DmpQ, DmpB's activity towards 3-vinylcatechol increased 2 fold (**Figure 3.6a**). Importantly, DmpQB was also more resistant to inactivation (**Figure 3.6b**) and cleaved approximately 2 fold more 3-vinylcatechol mg protein^{-1} prior to complete inactivation (**Figure 3.7**). After 28 hours, the CFU count of F1 (pDmpQB) was the highest, with 1.6 and 2 fold more CFU ml^{-1} than F1 (pDmpB) and F1 (pTodE), respectively. Given the C23O's initial rates of 3-vinylcatechol turnover (**Figure 3.6a**), these differences were expected. By 60 hours, the CFU ml^{-1} of F1 (pDmpB) had decreased nearly 17-fold. This decrease corresponded with the abrogation of DmpB C23O activity and subsequent accumulation of 3-vinylcatechol. The "catch-up" behavior in F1 (pTodE) growth is also consistent with the results presented in Fig. 6. Although TodE cleaved 3-vinylcatechol at a reduced rate compared to DmpQB, TodE was more resistant to

inactivation (**Figure 3.6b**) and eventually able to turnover greater amounts of substrate (**Figure 3.7**).

Catechols can initiate toxicity through a wide range of molecular mechanisms including the production of reactive oxygen species (Schweigert *et al.*, 2001), and it is likely that accumulation of 3-vinylcatechol following C23O inhibition had toxic consequences. While physiological endpoints were not a focus of this work, the noted decrease in CFUs in strains harboring the C23Os that were most prone to inactivation (**Figures 3.5 and 3.6b**) points to cell death and implicates 3-vinylcatechol as the probable cause of toxicity. In certain aromatic degradation systems, catechol toxicity is avoided by preferentially increasing the rate of catechol-consuming reactions through duplication of catechol dioxygenase genes. In this manner, catechol intermediates do not accumulate and potential toxicity is avoided. In *Ralstonia eutropha* JMP134(pJP4), it was shown that multiple copies of *tfdC* (chlorocatechol 1,2-dioxygenase) were required for efficient 3-chlorocatechol turnover and growth on 3-chlorobenzoic acid (Laemmli *et al.*, 2000; Perez-Pantoja *et al.*, 2003; Trefault *et al.*, 2004). In another study, the predominant organism in a chlorobenzene contaminated aquifer, *Ralstonia* JS745, was found to utilize a mosaic pathway containing partially duplicated *clcA* genes responsible for chlorocatechol degradation (Muller *et al.*, 2003; van der Meer *et al.*, 1998). Intriguingly, the upper pathway in JS745 was highly homologous to the genes encoding TDO (*todC1C2BAD)* in F1. Although both *tfdC* and *clcA* encode *ortho*- rather than *meta*- cleaving dioxygenases, these studies underscore the importance of efficient catechol consumption as a means for substrate expansion. In our current work, increasing the expression of TodE – thus mimicking gene duplication – was enough to confer growth on styrene and reduce 3-vinylcatechol-mediated toxicity. Interestingly, some bacteria capable of styrene metabolism appear to resolve the 3-vinylcatechol problem by avoiding its production

entirely. An alternative pathway for styrene degradation relies on a styrene monooxygenase and leads to phenylacetic acid (Mooney *et al.*, 2006). In this pathway, catechol is not produced and the hydroxylated ring is cleaved hydrolytically rather than oxidatively.

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CHAPTER 4

REDUCED CATECHOL PRODUCTION PERMITS PSEUDOMONAS PUTIDA F1 TO GROW ON STYRENE AND CONSTITUTES A NOVEL "LESS IS MORE" STRATEGY FOR GROWTH SUBSTRATE EXPANSION

ABSTRACT

The efficient turnover of catecholic intermediates during the metabolism of aromatic compounds is critical due to their unique ability to inactivate the dioxygenases responsible for their cleavage and cellular toxicity. 3-Vinylcatechol, for example, is a toxic metabolite that accumulates during styrene metabolism by *Pseudomonas putida* F1 and prevents F1 from growing on styrene. In this study, we characterized a spontaneous F1 mutant, designated SF1, which was capable of growth on styrene and which did not accumulate 3-vinylcatechol when exposed to styrene. Whereas adaptation to new aromatic substrates has typically been shown to involve increased or resistant catechol dioxygenase (C23O) activity, surprisingly, SF1 had wild type C23O activity, but grew more slowly on a related substrate, toluene. Resting cell assays suggested that the decreased growth rate of SF1 on toluene was due to SF1's reduced toluene dioxygenase (TDO) activity (≈50% of F1). DNA sequence analysis of SF1's *tod* operon revealed a single base pair mutation in *todA* (C479T), a gene encoding the reductase component of TDO. Replacement of the wild-type *todA* allele in F1 with $todA_{C479T}$ from SF1 reduced TDO activity, obviated vinylcatechol accumulation, and conferred the ability to grow on styrene. Collectively, our data demonstrate that slowing down the rate of vinylcatechol production via decreased TDO activity led to reduced C23O inactivation and was all that was required to permit growth on styrene. This novel "less is more" strategy sheds light on an alternative route that microbes have found to manage the

accumulation of toxic catecholic intermediates during the metabolism of aromatic compounds.

INTRODUCTION

The recent oil spill in the gulf of Mexico has served as cogent reminder of the importance of understanding the fate of petroleum hydrocarbons in the environment. Aromatic compounds comprise on average 15 % of crude oils and as much as 70 % of gasoline by volume (Hyne, 2001; Tessoro 2003), and while their degradation has been well studied for the last 40 years(Gibson *et al.*, 1968), there is still much to learn about the mechanisms bacteria employ to increase the range of aromatic compounds they can use for growth.

Bacterial aromatic hydrocarbon degradation pathways generally converge towards common intermediates such as protocatechuate, gentisate, hydroquinone, and catechol prior to ring cleavage by a dioxygenase (Gibson & Subramanian, 1984). Of these potential intermediates, catechols, produced during the biodegradation of numerous aromatic pollutants such as benzene and toluene, are perhaps the most common (Vaillancourt *et al.*, 2006). Catechol intermediates are enzymatically cleaved by either extradiol or intradiol dioxygenases, which cleave the ring at the 2,3- (*meta*) or 1,2- (*ortho*) position of the catechol, respectively (Harayama & Rekik, 1989). Extradiol dioxygenases (catechol-2,3-dioxygenases, C23Os) typically cleave a larger array of substrates and also occur in a wider range of biodegradation pathways (Vaillancourt *et al.*, 2006). Consequently, C23Os are generally recognized as the prototypical ring-cleavage enzymes.

C23Os may be inactivated during substrate cleavage, generally known as mechanism-based or suicide inhibition (Bartels *et al.*, 1984; Klecka & Gibson, 1981). Inactivation of this sort may lead to catechol accumulation and limit the range of

substrates available to a microorganism for growth (Cerdan *et al.*, 1995; Cerdan *et al.*, 1994; Klecka & Gibson, 1981; Ramos *et al.*, 1987; Rojo *et al.*, 1987; Vaillancourt *et al.*, 2002; Ward *et al.*, 2004).

Several studies have highlighted the deleterious effect of catechol accumulation during aromatic hydrocarbon metabolism (Park & Madsen, 2004; Park *et al.*, 2004; Perez-Pantoja *et al.*, 2003). In addition to suicide inhibition, catechols can initiate toxicity through a diverse range of molecular mechanisms, ranging from the production of reactive oxygen species to direct protein damage (Schweigert *et al.*, 2001a; Schweigert *et al.*, 2001b). Given this toxicity, bacterial cells have developed multiple strategies for preventing catechol accumulation. Previously described adaptations include expression of C23O-reactivating [2Fe-2S] ferredoxins (Cerdan *et al.*, 1994; Hugo *et al.*, 1998; Hugo *et al.*, 2000; Park *et al.*, 2002; Polissi & Harayama, 1993), recruitment of inactivation resistant C23Os (Cerdan *et al.*, 1994; Ramos *et al.*, 1987; Rojo *et al.*, 1987), and selection for chromosomal gene duplications which increase catechol consumption (Muller *et al.*, 2003; Perez-Pantoja *et al.*, 2003; van der Meer *et al.*, 1998). In the case of chlorocatechols, it has been demonstrated that cells must develop a delicate balance between catechol-producing and catechol-consuming reactions to avoid toxicity and permit growth on 3-chlorobenzoic acid (Perez-Pantoja *et al.*, 2003).

Recently, we found that C23O inactivation is the crucial factor which prevents *Pseudomonas putida* F1 from growing on styrene (see Chapter 3). Inactivation of TodE resulted in 3-vinylcatechol accumulation, toxicity, and cell death. Increasing the expression of TodE was sufficient to avoid catechol toxicity and permitted F1 to grow on styrene.

In this study, we describe a novel strategy that *P. putida* F1 mutants use to overcome catechol toxicity associated with styrene metabolism. Through a

combination of enzymatic assays and genetic analyses, we demonstrate that mutations leading to decreased toluene dioxygenase activity were sufficient to reduce production of 3-vinylcatechol and its associated deleterious effects, thereby permitting growth on styrene.

MATERIALS AND METHODS

Bacterials strains, plasmids, and growth conditions.

Pseudomonas putida F1, *Pseudomonas putida* SF1, F1 *todA*$_{C479T}$ and derivative plasmid-carrying strains were grown under gentle shaking at room temperature in Minimal Salts Medium (MSM) (Focht, 1994) containing suitable growth substrates. Toluene or styrene was provided in vapor phase for growth on agar plates or at a concentration of 5 mM in liquid media. Acetate was added at a concentration of 20 mM. Strains containing pBBR1MCS-5 and pKNGtodA$_{SF1}$ were selected on 25 µg ml^{-1} gentamicin or 50 µg ml^{-1} kanamycin, respectively. A list of strains and plasmids used in this study is presented in **Table 4.1**.

Cloning and DNA manipulation.

Primers for genes in the tod operon were designed based on the published genomic sequence of *Pseudomonas putida* F1 (GenBank Accession # NC_009512). A complete list of primers used in this study is presented in **Table 4.2**. PCR was performed using the HotStart Master Mix kit from Qiagen (Valencia, CA) and a PTC-200 DNA Engine thermocycler from MJ Research (Incline Village, NV). PCR fragments were digested with appropriate enzymes and ligated into pBBRMCS-5 (Gm^R) or pKNG101 (Str^R) using T4 DNA ligase (Promega, Madison, WI). The resultant plasmids were transformed into appropriate hosts by electroporation. Transformants were selected on LB plates containing antibiotics and screened by

PCR. All DNA sequencing was performed by the BioResource Center at Cornell University on an ABI3700 DNA Analyzer.

Isolation of styrene-adapted F1 (SF1).

P. putida F1 was grown overnight at room temperature in MSM acetate, washed twice in an equal volume of 30 mM KPO_4 buffer (PB), pH = 7.0, and serial diluted to 10^{-9} in PB. 5 µL volumes of each dilution were spotted onto MSM agar and exposed to styrene vapors. After ~ 10 days of exposure, mutant colonies capable of growing on styrene appeared at a frequency of 10^{-8}. One colony capable of robust growth was designated styrene-adapted F1 (SF1) and selected for further study. Three additional colonies (SF2 – SF4) were also selected for DNA sequencing, TDO activity, and C23O activity analyses (see below).

***todA* allele Replacement in F1.**

A 212 bp fragment of *todA* (nucleotides 3377 – 3588 of GenBank accession no. J04996) was PCR amplified from SF1 and cloned between the *Bam*HI and *Apa*I sites of pKNG101 (Kaniga *et al.*, 1991). DNA sequencing was used to confirm the presence of the C479T transition following transformation into *E. coli* BW21037 (Metcalf *et al.*, 1996) by electroporation. Since F1 exhibits background resistance to streptomycin, a kanamycin resistant derivative of pKNGtodA_{SF1} was generated in *E. coli* S17-1 λpir using the EZ-Tn5^{TM} <KAN-2> insertion kit (EpiCentre, Madison, WI). Biparental mating of S17-1 λpir (pKNGtodA_{SF1} Km^R) with F1 was used to generate Km^R F1 recombinants with pKNGtodA_{SF1} Km^R integrated into the chromosome. PCR with an upstream chromosomal primer (todB Forward) and a pKNG101 specific primer (pKNG MCS F) yielded amplicons in all tested colonies, confirming integration into *todA* as expected. Since plasmid integration knocked out

the F1 recombinants' ability to grow toluene, double crossover mutants were selected on MSM agar exposed to toluene vapor in the absence of Km. Colonies capable of growth on toluene appeared at a frequency of ~ 10^{-7} and were Km^S which confirmed loss of pKNGtodA_{SF1} Km^R. PCR was used to amplify the 212 bp *todA* region from putative double crossover mutants. These fragments were then digested with the restriction endonuclease Hpy99i, which only cut the wild-type allele. DNA sequencing was then employed to confirm the presence of the C479T mutation. A single confirmed double crossover mutant that had never been exposed to styrene, designated F1 *todA*$_{C479T}$, was used for further study.

Resting cell assays.

For resting cell enzymatic assays, F1 and relevant strains were grown to late exponential phase (OD_{600} = 0.8) at room temperature in MSM acetate and inoculated 1:100 into fresh MSM media containing acetate + toluene. Upon reaching late exponential phase, cells were harvested by centrifugation (10,000 x g for 10 min at 4 °C), washed twice in 30 mM PB, and resuspended in an equal volume of sterile buffer.

Measurement of C23O activity. C23O activity was measured as the rate of increase in A_{375} following the addition of 2 mM catechol as described previously (Salat-Trepat & Evans, 1971). Briefly, 200 µL volumes of resting cells in PB were assayed in triplicate using a Bio-Tek (Winooski, VT) Synergy^{TM} HT-I microplate reader. Following the addition of catechol, initial rates of *meta*-cleavage were calculated and correlated with OD_{600}. Concentrations of catechol *meta*-fission product were calculated using a reported extinction coefficient of 40 $mM^{-1}cm^{-1}$ (Seah *et al.*, 1998).

C23O inactivation assays. To quantify C23O inactivation following substrate cleavage, cultures were exposed to 2 mM of the various substrates. For 3-

vinylcatechol exposures, cultures were resuspended in filtered PB containing 50 µM of substrate. 3-Vinylcatechol was synthesized as previously described (Chapter 3). At set time points, 1 mL aliquots were taken, centrifuged at 16,000 × g for 1 minute, and resuspended in an equal volume of PB. C23O activity was measured as described above.

Measurement of toluene dioxygenase (TDO) activity. Toluene dioxygenase (TDO) activity was quantified using the indoxyl fluorescence method (Woo *et al.*, 2000). In resting whole cell cultures, TDO activity was measured as the rate of indoxyl formation following the addition of indole in *N*,*N*-dimethylformamide to a final concentration of 250 µM. Assays were performed in triplicate using excitation and emission wavelengths of 360 and 460 nm, respectively, with a Bio-Tek (Winooski, VT) Synergy™ HT-I microplate reader.

Table 4.1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
Strains		
P. putida F1	Cm^R, Amp^R; model organism containing the tod operon	(Zylstra *et al.*, 1988)
P. putida SF1	Styrene-adapted F1 mutant containing *todA* C479T allele	This study
F1 $todA_{C479T}$	F1 double recombinant containing $todA_{C479T}$ allele (naïve with respect to styrene)	This study
E. coli S17-1 λpir	λ*pir*; *hsdR pro recA*; RP4 2-Tc : : Mu-Km : : Tn7, pro, res^-, mod^+, Str^R, Trm^R	(Delorenzo & Timmis, 1994)
E. coli BW21037	DE3(lac)$_{X74}$ *uidA*(Δ*Mlu*I) : : *pir*-116 Tn5Lac4Cm^R $lacI_Q$ Δ$lacZ_{M15}$)*recA1*Δ*phnC?DEFGHIJKLMNOP*) 33–30 Δ*phoA532*	(Metcalf *et al.*, 1996)
Plasmids		
pKNG101	Suicide vector, *pir* $^-$ *oriR6K mobRK2 sacB* Str^R	(Kaniga *et al.*, 1991)
pBBR1MCS-5	Gm^R, broad host range expression vector	(Kovach *et al.*, 1995)
pPROBE	Km^R, broad host range vector for the creation of GFP promoter fusions	(Miller *et al.*, 2000)
pKNG $todA_{C479T}$	Derivative of pKNG101 containing a 212 bp fragment of $todA_{SF1}$; used to generate F1 $todA_{C479T}$	This study
pTodA	Derivative of pBBR1MCS-5 containing *todA* from F1; TodA expression vector	This study
pPROBE P_{todX}	GFP-transcriptional fusion containing P_{todX}	This study

Table 4.2. PCR primers used in this study for DNA amplification and cloning. All primers were designed and ordered through IDT PrimerQuest™. PCR was performed as described in the text. Enzyme restriction sites used for cloning are highlighted.

Name	Sequence	Characteristics
TodE Forward	5' – ACA **GGG CCC** ACA AGC ACT TCG GTT GAG ACA GGA – 3	**ApaI**
TodE Reverse	5' – ACA **GGA TCC** TGG CAT ATG GGC ATC CAG GTA GAA – 3'	**BamHI**
TodA Forward	5' – ACA **GGG CCC** TTC CCA ATC AAG GTC GAA GGC GAT – 3'	**ApaI**
TodA Reverse	5' – ACA **GGA TCC** TTC CGC GAC ATA ACG ATC CAC GAT – 3'	**BamHI**
$TodA_{pKNG}$ Forward	5' – ACA **GGG CCC** CAG GTA TTG CGC GAT AGT TGG ACT – 3'	**ApaI**
$TodA_{pKNG}$ Reverse	5' – ACA **GGA TCC** AAC TCG ACC TGC ACA CCA AGT TCT – 3'	**BamHI**
TodB Forward	5' – ACA **GGG CCC** ACC ATC CTG GCC AAT AAC CTC AGT – 3'	**ApaI**
TodB Reverse	5' – ACA **GGA TCC** TTG GAC AAG GAT GGT CGG TCA TAG – 3'	**BamHI**
TodC1 Forward	5' – ACA **GGG CCC** GTT CTG AAA GCA CGT CAT CGG CAA – 3'	**ApaI**
TodC1 Reverse	5' – ACA **GGA TCC** TTT CAA ATG GAC AAG GTC CAC CGC – 3'	**BamHI**
TodC2 Forward	5' – ACA **GGG CCC** ATG CGG TGG ACC TTG TCC ATT T– 3'	**ApaI**
TodC2 Reverse	5' – ACA **GGA TCC** TGG ACA TAC ATA TTG CGG CAG GGT – 3'	**BamHI**
TodD Forward	5' – ACA **GGG CCC** CGG CTG GCA GAT CTT TCA AAC AGT – 3'	**ApaI**

Table 4.2 (Continued)

TodD Reverse	5’ – ACA **GGA TCC** AGC CGA AGG TAG CCC AAT CTT TGA – 3’	**BamHI**
TodF Forward	5’ – ACA **GGG CCC** GTT GCG GCA AAG CCA CAG TCA AAT– 3’	**ApaI**
TodF Reverse	5’ – ACA **ATC GAT** TGA CGT GCT TTC AGA ACG GAT GGT– 3’	**ClaI**
TodS Forward	5’ – ACA **GGG CCC** ATT CTC AGG ATG ATA CGA GGG CGT– 3’	**ApaI**
P_{todS} Forward	5’ – ACA **GGG CCC** TTT CAG GTC GTC CGT AGT TGC TCA – 3’	**ApaI**
P_{todS} Reverse	5’ – ACA **GGA TCC** TCA CAC GTC AGC TCT TCA GAT CCT – 3’	**BamHI**
TodT Reverse	5’ – ACA **GGA TCC** TGA CCA CGA AAC CAT GCC AGA GTA – 3’	**BamHI**
TodX Forward	5’ – ACA **GGG CCC** GGC GGT GTT TGC CTA CTT CAC TTT – 3’	**ApaI**
TodX Reverse	5’ – ACA GGA TCC ACC ATG AAC GAG TAC CAC AGG GTT – 3’	**BamHI**
P_{todX} Forward	5’ – ACA **GCA TGC** CGA AAG CCG CAA AGT GCT TAG GTT – 3’	**Sph1**
P_{todX} Reverse	5’ – ACA **GGG CCC** AAA GTG AAG TAG GCA AAC ACC GCC– 3’	**BamHI**
pKNGMCS F	5’ – AAG GAT TTG CAG ACT ACG GGC CTA – 3’	

RESULTS

SF1 resists C23O inactivation following styrene exposure, but is equally susceptible to inactivation by 3-vinylcatechol.

Previously we found that F1 accumulates toxic levels of 3-vinylcatechol and loses catechol dioxygenase (C23O) activity when exposed to styrene (see Chapter 3). Intriguingly, we noticed that SF1, an F1 mutant capable of robust growth on styrene, did not accumulate 3-vinylcatechol (data not shown) and retained C23O activity when exposed to styrene (**Figure 4.1**). Based on our previous work and that of others describing growth on compounds like chlorinated biphenyls and chlorobenzenes (Perez-Pantoja *et al.*, 2003; Vaillancourt *et al.*, 2002; Vaillancourt *et al.*, 2005), we initially hypothesized that the lack of 3-vinylcatechol accumulation in SF1was due to either an increase in C23O activity or to the acquisition of 3-vinylcatechol resistant C23O activity. Surprisingly, however, the level of C23O activity of SF1 was the same as that of F1 and was just as susceptible to inactivation by 3-vinylcatechol (**Figure 4.1**). DNA sequencing also revealed that *todE* in SF1 was 100 % identical to F1 (data not shown).

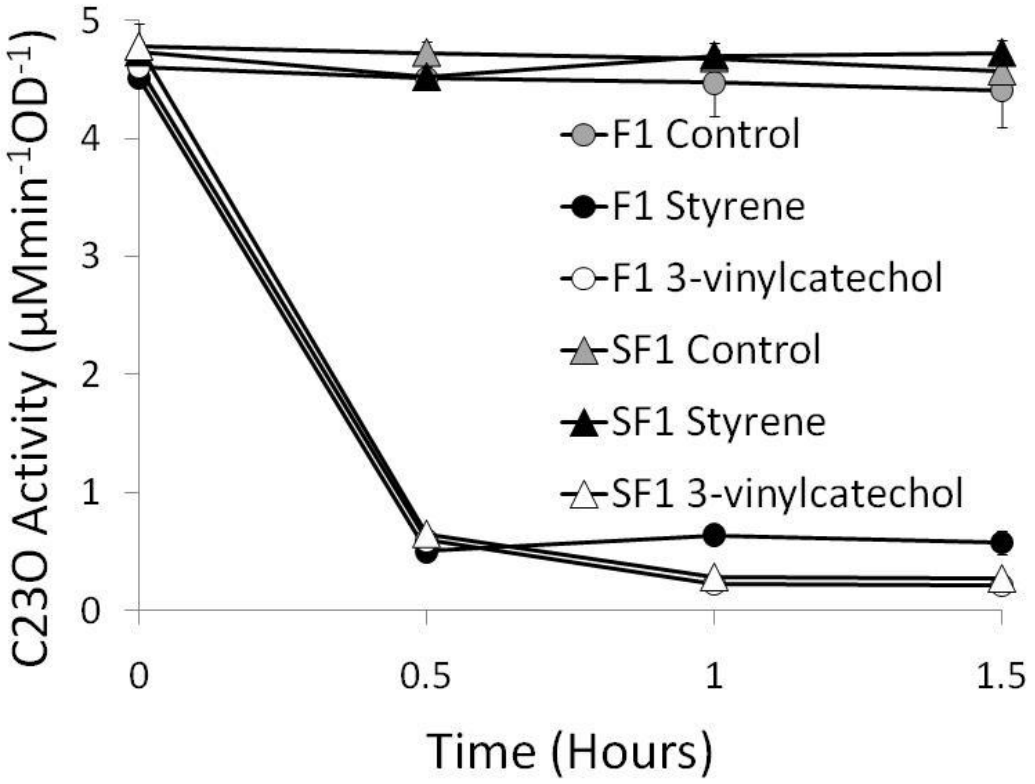

Figure 4.1. **C23O Activity in F1 and SF1 following exposure to styrene and 3-vinylcatechol.** In F1, C23O activity was abolished when exposed to both 2 mM styrene and 50 µM 3-vinylcatechol. In SF1, only 3-vinylcatechol caused C23O inactivation. C23O activity was measured following the addition of 2 mM catechol as described in methods. Error bars represent 1 standard deviation (n = 3).

SF1 experiences a lag when grown on toluene

These preliminary observations suggested that SF1 might be employing a different strategy to overcome 3-vinylcatechol toxicity. SF1's slow growth on toluene, but not on acetate, provided the first hint as to what this might be (**Figure 4.2**). This discrepancy in growth suggested that there might be a mutation in the tod pathway that affected both toluene and styrene metabolism in SF1.

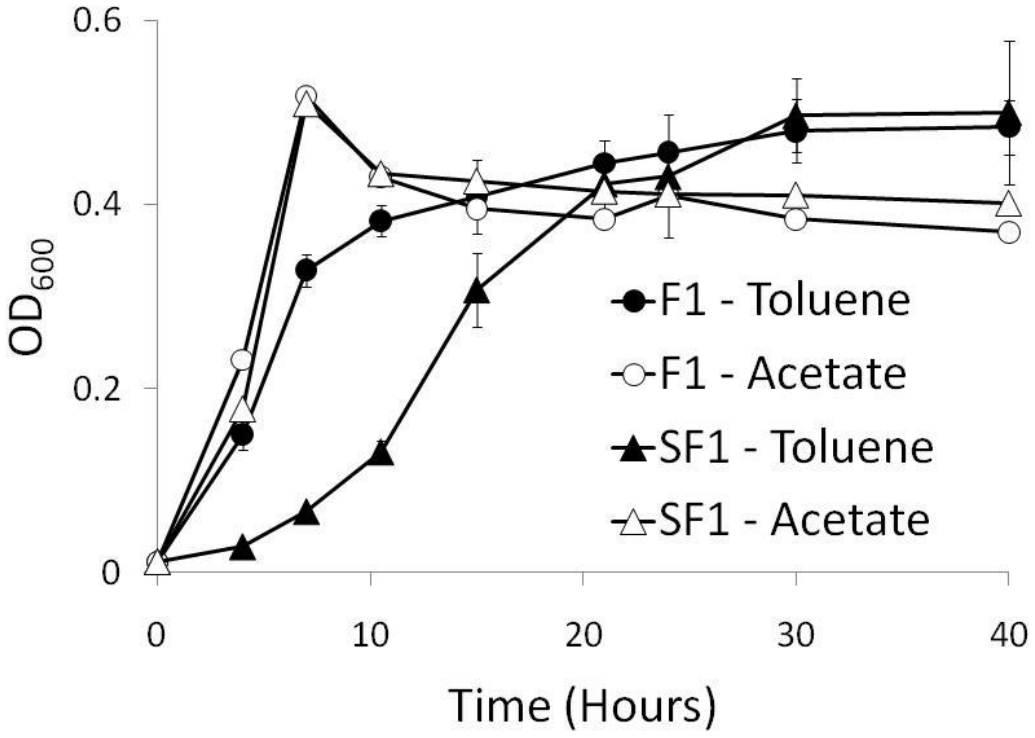

Figure 4.2. Growth of F1 and SF1 on toluene and acetate. When grown on 2 mM toluene, growth of SF1 appeared delayed by approximately 10 hours when compared to F1. When grown on 2 g l^{-1} acetate, no lag was observed. Error bars represent one standard deviation (n = 3).

TDO Activity is reduced in SF1, but C23O activity is identical.

The similarity in C23O activity between F1 and SF1, along with the lag in toluene-grown SF1, suggested the potential involvement of an enzyme upstream of the C23O. In the tod pathway, transformation of styrene into 3-vinylcatechol is catalyzed by the multi-component toluene dioxygenase (TDO), encoded by *todC1C2BA*, and *cis*-toluene dihydrodiol dehydrogenase, encoded by *todD* (Zylstra & Gibson, 1989). These enzymes catalyze the hydroxylation and re-aromatization of the ring, respectively. A simple fluorescence assay (Woo *et al.*, 2000) was therefore used to determine if there was a difference between the TDO activity in F1 and SF1 following induction with toluene. As **Figure 4.3** demonstrates, there was nearly a 2-fold decrease in TDO activity in SF1.

To ensure that this variation was not due to differential induction of tod pathway enzymes, C23O activity was measured and found to be identical in both strains (**Figure 4.3**). Transcriptional regulation of the tod operon was also investigated using a P_{todX} : GFP reporter construct and found to be identical for F1 and SF1 (**Figure 4.4**). DNA sequencing of key regulatory genes and promoters in SF1 including *todS, todT,* and *todX* were also found to be 100 % identical to those of F1.

In a separate assay, three additional styrene-adapted F1 mutants were analyzed for TDO activity along with F1 and SF1 as described above. Intriguingly, all tested strains exhibited reduced TDO activity relative to F1 while retaining comparable C23O activity. Ratios of TDO activity/C23O activity for SF1 and additional mutants relative to F1 are presented in **Table 4.3**.

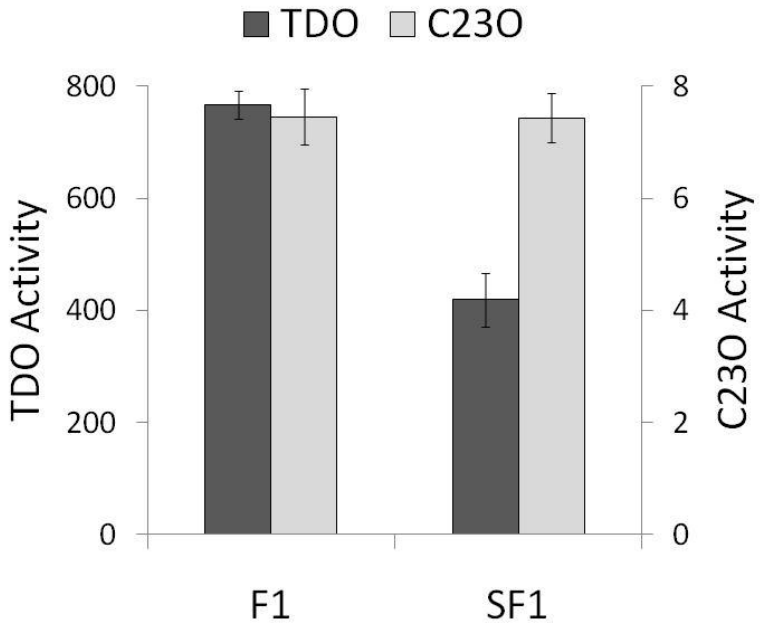

Figure 4.3. TDO vs. C23O Activity in F1 and SF1. Resting cultures of SF1 exhibited significantly reduced TDO activity compared to F1. C23O activity was identical in both strains. Units of TDO and C23O activity were fluorescence min^{-1} OD^{-1} and μM min^{-1} OD^{-1}, respectively. Activities were normalized to OD_{600}. Error bars represent one standard deviation (n = 3).

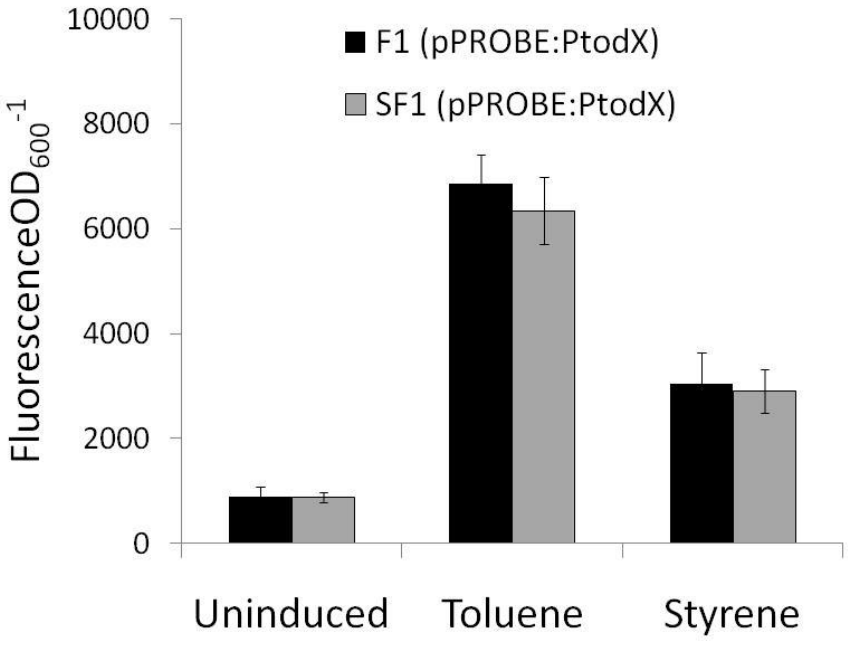

Figure 4.4. Induction of the *tod* operon by toluene and styrene. Transcriptional behavior of the *tod* operon appears to be identical in F1 and SF1 as measured by GFP fluorescence. Error bars represent one standard deviation (n = 3).

Table 4.3. Ratios of TDO / C23O Activity in SF1 mutants

Strain	F1	SF1	SF2	SF3	SF4
TDO activity / C23O activity[a]	1[b]	0.42 ± 0.11	0.38 ± 0.08	0.39 ± 0.13	0.58 ± 0.14

[a] TDO activity was measured as fluorescence min^{-1} OD^{-1}, while C23O activity was measured as μM min^{-1} OD^{-1}

[b] The TDO/C23O ratio in F1 was standardized to 1 for relative comparison to other SF1 strains. Differences in C23O activity between strains were statistically insignificant ($P<0.05$).

There is a single nucleotide polymorphism in *todA* from SF1.

The reduced TDO activity in SF1 suggested the existence of one or more mutations in *todC1C2BA*, the genes encoding the components of TDO. Sequencing of *todC1*, *todC2*, and *todB* revealed no nucleotide changes compared to F1 (data not shown). Sequencing of *todA*, which encodes the reductase component of TDO (TDO-R), however, revealed the existence of a single C to T transition at position 479 from the *todA* start codon in Accession No. J04996 (Zylstra & Gibson, 1989). In the folded TDO-R protein, this would have resulted in the substitution of methionine for threonine at residue 160 (pdb reference 3ef6) (Friemann et al 2009). The result of *in silico* substitution of T160M is presented in **Figure 4.5**.

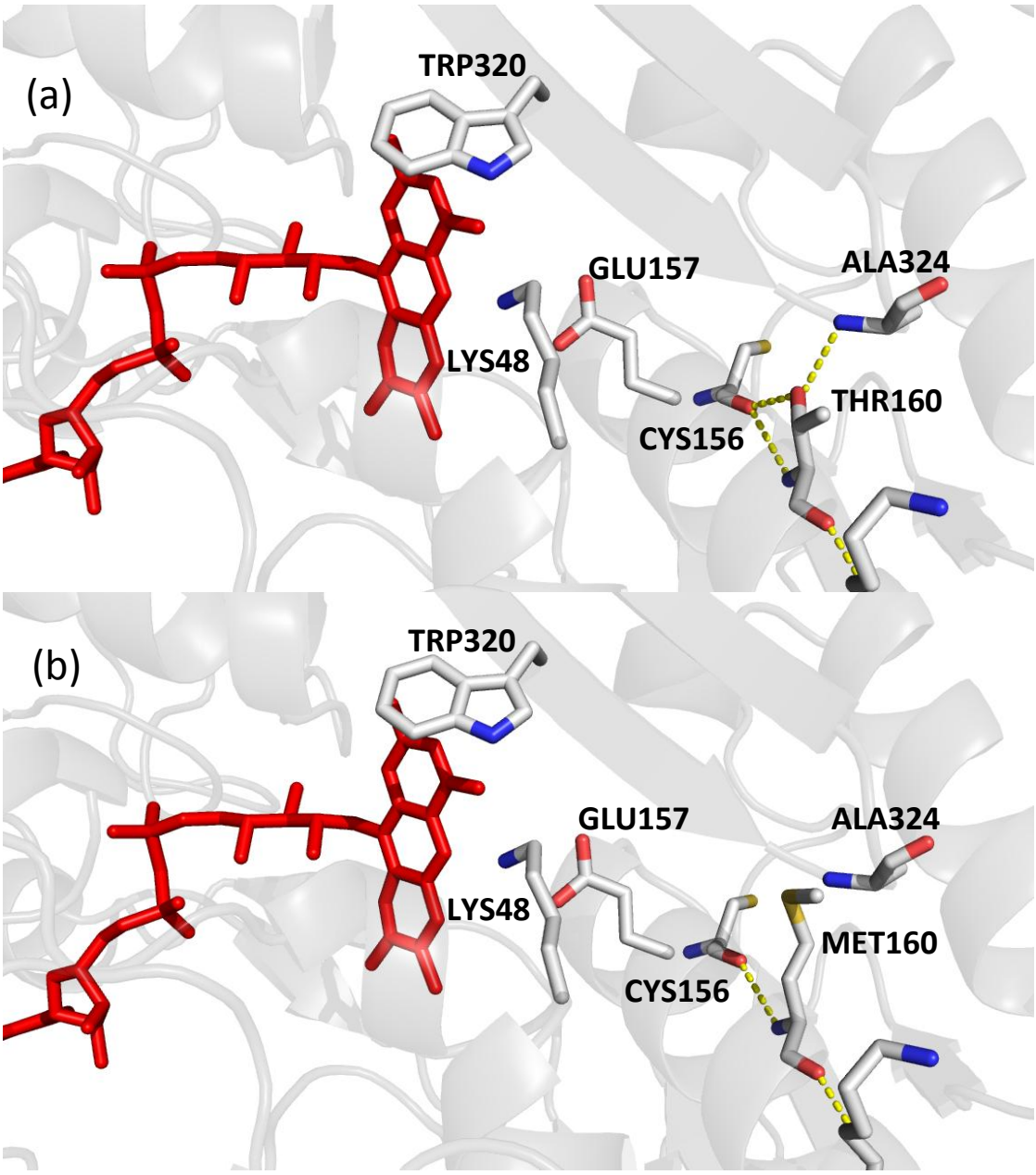

Figure 4.5. Substitution of THR160 with MET160 in TodA$_{SF1}$. In TodA$_{SF1}$, THR160 (**a**) was replaced with MET160 (**b**), likely preventing hydrogen bonding with Cys156 and Ala324. Putative residues involved in hydride transfer (LYS48 and GLU157) and TDO-F docking (TRP320) are labeled. FAD is shown in red. Structure of TodA was based on PDB reference 3ef6 (Friemann *et al.*, 2009). This figure was produced using *PyMOL* (http://www.pymol.org).

Introduction of *todA*$_{C479T}$ into F1 confers reduced TDO activity, the ability to grow on styrene, and decreased C23O inactivation after styrene exposure.

To determine if the *todA*$_{C479T}$ allele permitted SF1 to grow on styrene and caused its reduced TDO phenotype, the mutant allele was introduced into the F1 chromosome through a double crossover, generating F1 *todA*$_{C479T.}$

TDO activity in F1*todA*$_{C479T}$ was nearly identical to SF1 (**Figure 4.6a**), as was the observed growth lag on toluene (**Figure 4.6b**). We also tested the corollary of this hypothesis by introducing a wild type copy of *todA* (pTodA) into SF1. The expression of WT TodA in SF1 (pTodA) increased TDO activity, although it did not completely restore it to F1 levels. The lag during growth on toluene, however, was eliminated due to (pTodA) expression.

The presence of the *todA*$_{C479T}$ allele in F1 also affected the level of C23O activity following styrene exposure: after a 1 hour exposure to styrene, C23O activity in F1 *todA*$_{C479T}$ was still more than 90 % of that of an unexposed control (**Figure 4.7a**). Compared to F1, this represented a 7-fold increase in C23O activity. SF1 (pTodA), which expressed both mutant and WT *todA* alleles, exhibited a 2.5-fold reduction in C23O activity compared to the parent strain. Despite the difference in sensitivity to styrene, the C23O activity of all strains was equally susceptible to 3-vinylcatechol.

When resting F1 cells were exposed to styrene, 3-vinylcatechol accumulated in the culture media and polymerized to form an opaque, brown product (Chapter 3). The appearance of this product acted as a qualitative indicator of C23O inactivation and subsequent catechol accumulation. To facilitate a simple visual comparison between metabolite accumulation in F1*todA*$_{C479T}$, SF1 (pTodA), and parent strains, each strain was streaked onto MSM Acetate agar and exposed to styrene vapor. After 48 hours, the accumulation of brown product was obvious in F1 and SF1 (pTodA),

and was indicative of 3-vinylcatechol accumulation due to C23O inactivation (**Figure 4.7b**). In both SF1 and F1*todA*$_{C479T}$, only the yellow *meta*-fission product 6-vinyl HODA was evident, even following extended (>72 hrs) incubations with styrene.

As expected given its C23O inactivation behavior, F1*todA*$_{C479T}$ was capable of growth on styrene as a sole carbon source, although not as robust as that of SF1 (**Figure 4.8**). This suggested the probable existence of additional mutation(s) in SF1. Growth of SF1 (pTodA) on styrene was impeded, but not entirely eliminated despite the accumulation of polymerized 3-vinylcatechol. Although this growth may be partially attributed to residual C23O activity following styrene exposure (**Figure 4.6a**), it could also be due to uncharacterized mutations which increase SF1's viability when exposed to 3-vinylcatechol or alter metabolite formation within the tod pathway.

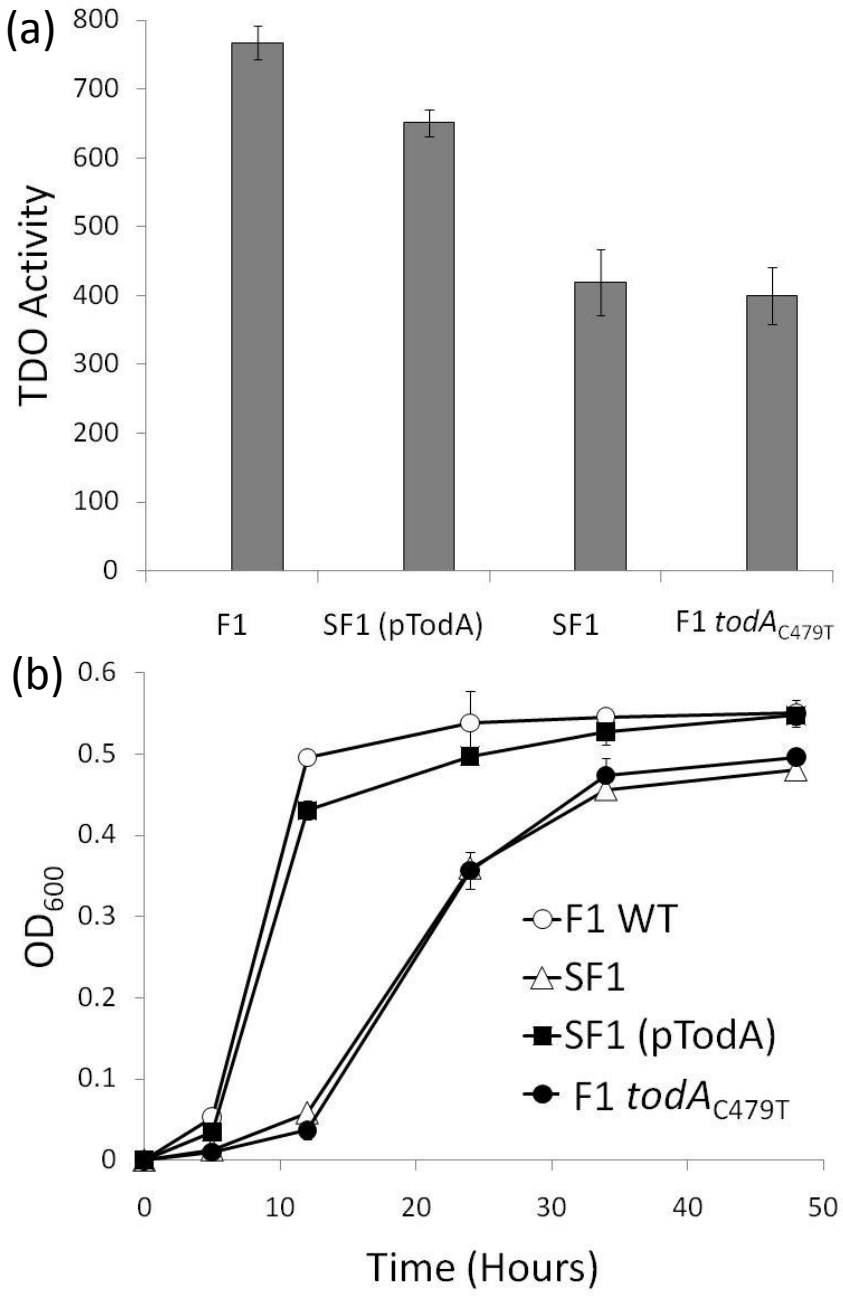

Figure 4.6. TDO activity and corresponding growth on toluene following allele replacement. (a) In F1 C479T, TDO activity was reduced in SF1 levels due to introduction of the mutant *todA* allele. SF1 (pTodA) showed increased TDO activity, nearly matching F1 in magnitude. (**b**) The presence of a lag in growth on toluene correlated with reduced TDO activity. F1*todA*$_{C479T}$ mimicked the behavior of SF1, while SF1 (pTodA) nearly matched the growth kinetics of F1. In both (**a**) and (**b**), error bars represent one standard deviation (n = 3).

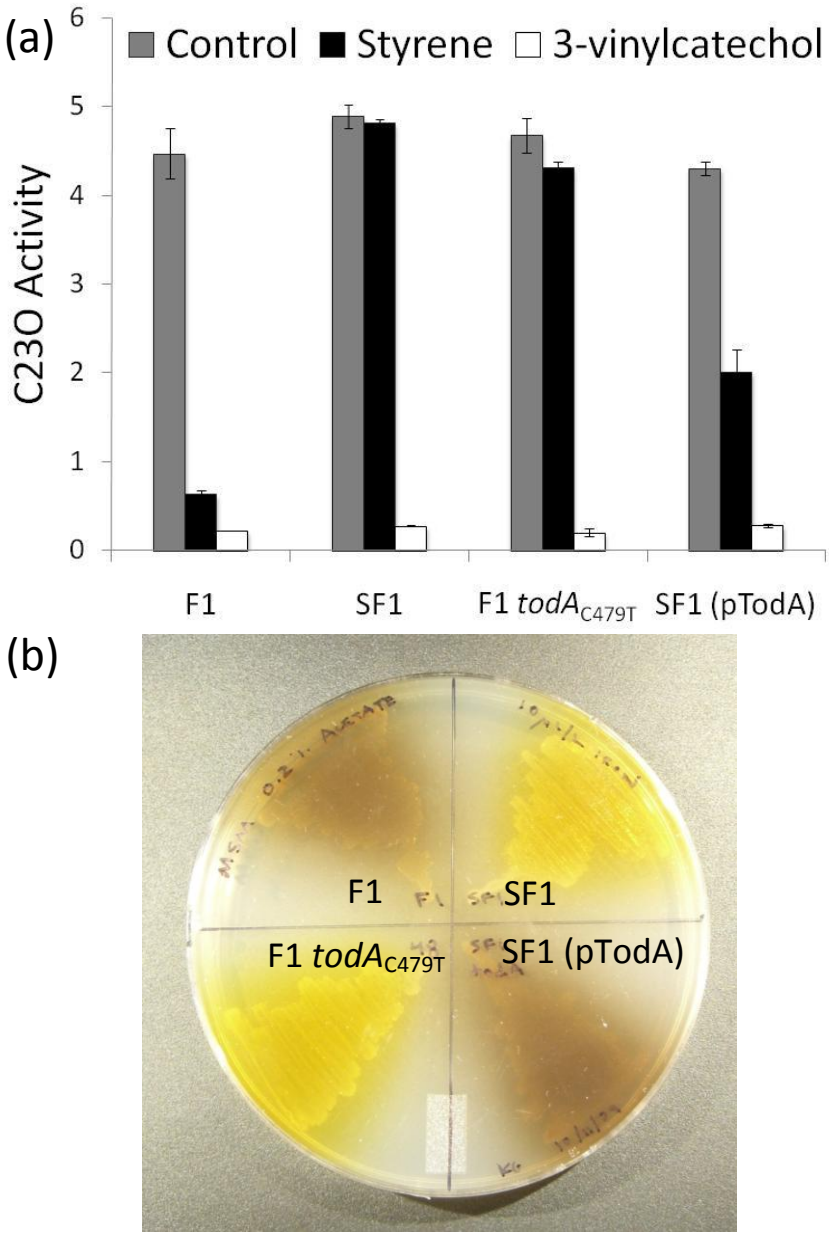

Figure 4.7. C23O inhibition following exposure to styrene and 3-vinylcatechol. (**a**) As expected, F1*todA*$_{C479T}$ was resistant to C23O inactivation following exposure to styrene, but not 3-vinylcatechol. SF1 (pTodA) experienced increased C23O inactivation following styrene exposure relative to SF1. Error bars represent one standard deviation (n = 3). (**b**) Following exposure to styrene, F1 and SF1 (pTodA) accumulated 3-vinylcatechol, while SF1 and F1 *todA*$_{C479T}$ exhibited transient accumulation of the yellow *meta*-fission product, 6-vinyl HODA. 3-Vinylcatechol accumulation correlated with C23O inactivation following styrene exposure (**a** and **b**). Strains were grown on MSM acetate agar and incubated with styrene vapors.

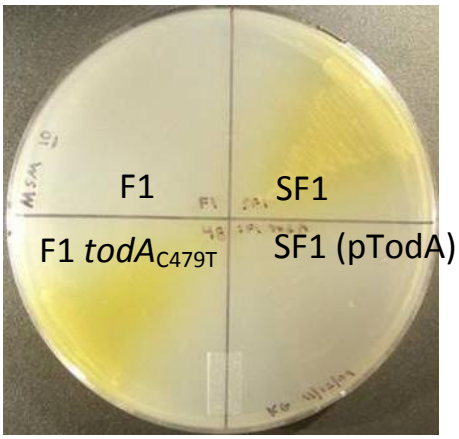

Figure 4.8. Growth of strains on MSM agar + styrene. F1 *todA*$_{C479T}$ was capable of growth on styrene as a sole carbon source. Growth of SF1 (pTodA) was inhibited. Strains were grown for 48 hours on MSM agar + styrene vapor.

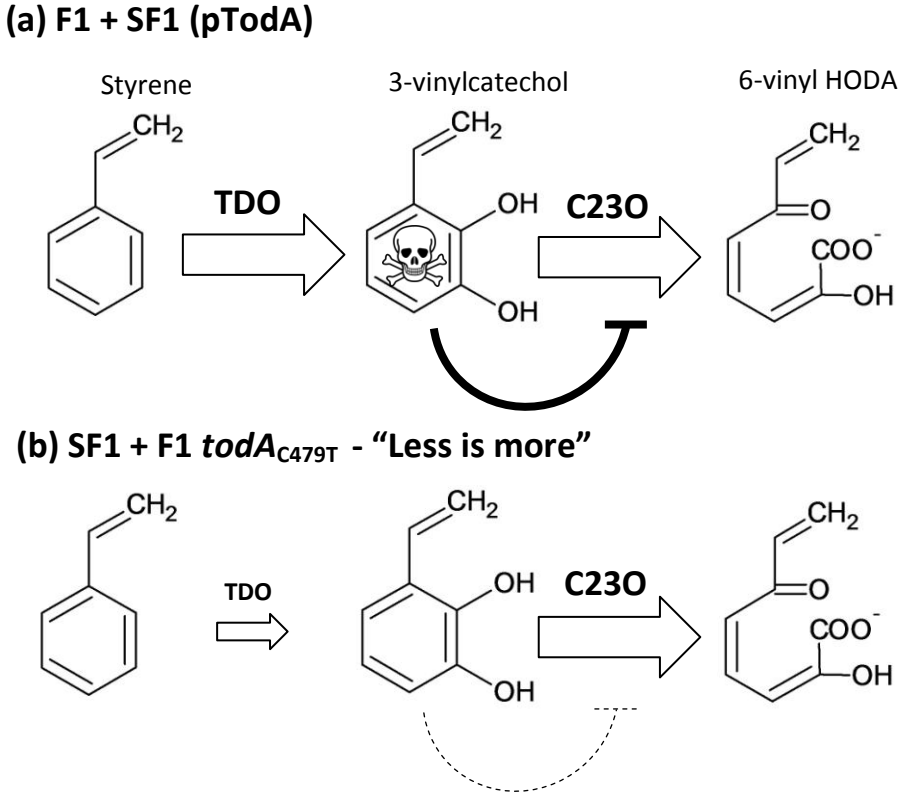

Figure 4.9. 3-Vinylcatechol production and consumption in F1 and SF1 (pTodA) vs. SF1 and F1 *todA*$_{C479T}$. In F1 and SF1 (pTodA), TDO rapidly produces 3-vinylcatechol, resulting in abolition of C23O activity and the rapid accumulation of catechol (**a**). SF1 and F1 *todA*$_{C479T}$ both utilize the "less is more" strategy. The mutant *todA*$_{C479T}$ allele results in lowered TDO activity, reduced production of 3-vinylcatechol, reduced C23O inactivation, and the capability to grow on styrene.

DISCUSSION

In this study, we characterized a novel mutant of *Pseudomonas putida* F1, named SF1, with reduced toluene dioxygenase activity that was capable of growing on styrene. The main consequence of reduced TDO activity was slower production of the toxic intermediate 3-vinylcatechol. This prevented C23O inactivation and more generalized catechol-related toxicity. Even though we have previously shown that over-expression of TodE increased 3-vinylcatechol consumption and allowed F1 to grow on styrene (Chapter 3), the monocistronic nature of the tod operon, in which the upper and lower pathways are transcribed as a single unit, meant that overproduction of C23O activity via increased gene dosage was unlikely. Rather, SF1 achieved the same end – balanced production and consumption of toxic catecholic intermediates – by reducing TDO activity while maintaining wild-type levels of C23O activity (**Figure 4.9b**).

TDO activity in SF1 was reduced as consequence of the C479T transition in *todA*, which is expected to result in the replacement of Thr160 with Met160 in $TodA_{SF1}$. This reduction in activity was likely caused by subtle structural changes in regions associated with electron transfer and docking of the ferredoxin component of the TDO holoenzyme. The ferredoxin (encoded by *todB*) shuttles electrons from the reductase (*todA*) to the terminal dioxygenase (*todC1C2*), with the latter ultimately catalyzing the addition of dioxygen to the aromatic ring to form a dihydrodiol (Gibson *et al.*, 1970).

TodA is actually comprised of 3 domains: an FAD binding domain (residues 1-108 and 239-317), an NADH-binding domain (residues 109-238), and a C-terminal domain (residues 318-410) (Friemann *et al.*, 2009). Lys48 and Glu157 have been suggested to play conserved roles in electron transfer from NADH to FAD, while Trp320 of the C-terminal domain has been suggested to mediate electron shuttling

from FAD to the Reiske center of TodB, the ferredoxin (Friemann *et al.*, 2009). In $TodA_{T160M}$, replacement of Thr160 with Met160 would be expected to prevent the formation of two hydrogen bonds from threonine's side chain hydroxyl to the main chain oxygen of Cys156 and nitrogen of Ala324, respectively (**Figure 4.5**). Cys156 is located within the α-helix containing the putative catalytic residue Glu157, while Ala324 resides adjacent to the β-sheet containing Trp320. Although Thr160 does not interact with Glu157 or Trp320 directly, the dissolution of nearby hydrogen bonds following substitution to Met160 is likely to have subtly altered the position of these key catalytic residues.

These results contrast markedly with those observed during adaptation to other aromatic hydrocarbons where gene dosage or increased transcription of catabolic enzymes permitted bacteria to grow on new substrates (Perez-Pantoja *et al.*, 2003). Thus, although previous studies have also underscored the need for efficient catechol turnover in aromatic degradation pathways, particularly in the case of chlorinated catechol intermediates (Muller *et al.*, 2003; Perez-Pantoja *et al.*, 2003; van der Meer *et al.*, 1998), the organisms studied achieved this in a much different manner than SF1.

For example, while working with *Ralstonia eutropha* JMP134(pJP4), Perez-Pantoja *et al*. (2003) found that multiple copies of *tfdC*, encoding chlorocatechol 1,2-dioxygenase, increased catechol turnover and were necessary for growth on 3-chlorobenzoate. Mutants lacking multiple copies of *tfdC* were unable to grow on 3-chlorobenzoate and were subject to toxicity due to the accumulation of chlorocatechols. Importantly, the authors noted that increasing the rate of chlorocatechol production through the heterologous expression of a broad substrate toluate dioxygenase (*xylS-xylXYZL*) had a negative effect on growth. Their work made it clear that efficient growth on 3-chlorobenzoate required the proper gene dosage and a delicate balance between chlorocatechol-producing and chlorocatechol-

consuming reactions. This need for proper gene dosage has likely played a key role in the genetic organization and evolution of catabolic pathways for chloroaromatic degradation and lead to the separation of so called "upper pathways" that hydroxylate the ring, and "lower pathways" which cleave it (Laemmli *et al.*, 2000; Muller *et al.*, 2003; Trefault *et al.*, 2004; van der Meer *et al.*, 1998).

With respect to transcription, Choi *et al.* (2003) demonstrated that adaptation of F1 to growth on biphenyl, *n*-propylbenzene, and *n*-butylbenzene required mutations in *todS* as well as *cymR*, allowing for induction of the tod operon and recruitment of CmtE as an alternative catabolic enzyme, respectively. In the closely related strain *Pseudomonas putida* KL47, mutations in *todS* which increased transcription of the *tod* pathway were again required for catabolic adaptation to substrates such as biphenyl (Lee *et al.*, 2006). In the current study, however, it is perhaps not surprising that tod operon regulation was identical in SF1 and F1, as upregulation of beneficial C23O activity would also have increased deleterious TDO activity. This is because the entire catabolic *todXFC1C2BADEGIH* operon is transcribed from a single promoter designated P_{todX} and mediated by TodS and TodT, (Busch *et al.*, 2007; Lacal *et al.*, 2006; Lacal *et al.*, 2008; Lau *et al.*, 1997; Mosqueda *et al.*, 1999). Consequently, upregulation would leave the ratio of TDO / C23O activity unchanged and have no effect on catechol turnover (**Figure 4.9a**).

Our work with styrene suggests that the coupled regulation of the upper and lower pathways in the tod operon might be one factor limiting growth substrate range and suggests that a modular strategy for control of the upper and lower pathways might confer selective advantages on organisms trying to exploit diverse aromatic substrates for growth. Interestingly, in many Shingomonads which are known for their ability to degrade a wide range of aromatic substrates, the upper and lower pathways appear as distinct modules and are not coordinately regulated. In many

cases they are not even found in the same region of the genome (Armengaud *et al.*, 1998; Kim & Zylstra, 1999).

In the absence of distinct upper and lower pathways, SF1 was forced to find another way to alter the ratio of TDO to C23O activity. It accomplished this by taking advantage of the less active TDO encoded by the C479T allele of *todA*. Although F1 *todA* $_{C479T}$ could clearly grow on styrene, it did not grow as quickly as SF1. This observation, coupled with the fact that SF1 (pTodA) was capable of modest growth on styrene, suggested that SF1 might contain additional mutations. That said, the beneficial impact of reduced TDO activity on styrene metabolism is unambiguous and was all that was required to allow growth on styrene. The presence of diminshed TDO activity in three additional SF1 mutants – none of which contained mutations in *todA* – underscores the critical importance of reducing 3-vinylcatechol production as a means of avoiding C23O inactivation and subsequent toxicity. While the "less is more" strategy described in this work was only demonstrated in response to styrene, it is likely that this strategy for alleviating C23O inactivation will permit adaptation to other substrates which also produce inactivating catechols and is yet another reminder of the need for regulating the production and consumption of this toxic intermediate.

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