MOLECULAR GENETIC AND PHYSIOLOGICAL STUDIES TO UNRAVEL
THE MYSTERY OF SPHINGOMONAS WITICHII RW1
DIBENZO-P-DIOXIN DEGRADATION

By

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ABSTRACT OF THE DISSERTATION

Molecular Genetic and Physiological Studies to Unravel the Mystery of Sphingomonas wittichii RW1 Dibenzo-p-dioxin Degradation

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Dibenzofuran and dibenzo-p-dioxin are ubiquitous environmental pollutants in soil and sediment. Sphingomonas wittichii RW1 is one of a few strains known for the ability to grow on the related compounds dibenzofuran (DBF) and dibenzo-p-dioxin (DXN) as the sole source of carbon. The genes for the initial steps in the DBF catabolic pathway (ring hydroxylating dioxygenase, ring cleavage dioxygenase, and a hydrolase) which result in the formation of salicylate and a five-carbon fragment have been localized to a mega-plasmid designated pSWIT02 in RW1. Plasmids highly similar to pSWIT02 have been found in other DBF degrading Sphingomonas strains. However, despite having the pSWIT02-encoded DBF degradation pathway these other bacteria are not capable of growth on DXN. This thesis describes involvement of chromosomally encoded genes in dibenzo-p-dioxin degradation by RW1. RW1 lacking the pSWIT02 dbfB gene grows extremely slowly on DBF and accumulates the ring cleavage substrate 2,2',3-trihydroxybiphenyl. The mutant grows normally on DXN as the sole source of carbon indicating that dbfB is not necessary for the DXN catabolic pathway and suggesting involvement of other ring cleavage dioxygenases in DXN pathway. Knockout of gene
SWIT3046 resulted in a strain that grows normally on DBF but that does not grow on DXN. The double knockout does not grow on either DBF or DXN. These results prove that separate ring cleavage enzymes are necessary for DBF and DXN degradation.

We then examined the third enzymatic step in RW1, the hydrolase. RW1 lacking the pSWIT02 encoded gene $dxnB1$ or the chromosome encoded gene $dxnB2$ grow normally on both DBF and DXN. A double knockout of both genes does not grow on DBF but still grows on DXN. We then examined previously published transcriptomic data that showed that the SWIT0910 encoded hydrolase is up regulated during growth on DBF and DXN. A knockout of SWIT0910 grows normally on DBF but does not grow on DXN. Our results demonstrate that a chromosomally encoded hydrolase, SWIT0910, is absolutely required for growth on DXN and that two different hydrolases (chromosomally and plasmid encoded) contribute equally to growth on DBF.

Genes for three biphenyl ring cleavage dioxygenases from *Burkholderia xenovorans* LB400, *Sphingomonas yanoikuyae* B1, and *Pseudomonas putida* F1 were moved into a mutant lacking the RW1 DBF and DXN ring cleavage genes. All three ring cleavage dioxygenases allowed the mutant RW1 to grow on DBF at different rates. Interestingly, only $bphC$ from *Burkholderia xenovorans* LB400 allowed RW1 mutant to grow on DXN.
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Chapter 1: Introduction

Dioxins

Dioxins are a group of compounds discovered as trace impurities in various other aromatic compounds. Polychlorinated dibenzo-\textit{p}-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are the major group of dioxins in addition to some polychlorinated biphenyls (PCBs). They have similar chemical structures and are chemically classified as halogenated aromatic hydrocarbons (1). They accumulate in the environment as unwanted by-products and are often considered xenobiotic compounds, through the accidental release from polychlorinated biphenyl (PCBs), pentachlorophenol (PCP), or herbicide production and the improper disposal of many organic wastes (2-4). Combustion of organic compounds in the presence of chlorines is one of the major sources for PCDDs/PCDFs such as forest fires, municipal waste incineration, and from pulp and paper processing (3, 5, 6). They were first reported in incinerator fly ash samples in 1977 and 1978 and they have come to public attention after the Seveso accident in Italy in 1976 (7).

These aromatics cause public concern because they are globally distributed and accumulate in all environmental media as recalcitrant pollutants and are highly toxic for the biosphere. PCDDs and PCDFs are chemically stable in the environment, have a low solubility in water, and accumulate in the food chain mainly in high fat foods such as animal fats (3).

The primary sources of human exposure to polychlorinated dioxins are through combustion and through diet including fish, dairy products, and meat (4). Dioxins consists of two benzene rings connected by one or two oxygen atoms (Figure 1).
There are 210 PCDDs/PCDFs with chlorine substituents and seventeen of them, mainly those that having chlorines substituted in the 2,3,7,8 positions, having high toxicity and posing a risk to human health represented by carcinogenic effects, immunological effects, and pose potential effects on animal reproductive systems (8, 9). 2,3,7,8-TCDD (Figure 1C) is the most extensively studied congener which is lipophilic, insoluble in water, and has a very low volatility (10). It is the most toxic congener of this group of compounds and many studies have shown the toxic effects of this compound to be fatal for specific species of animals in low concentrations (3, 7). It has a lethal dose of 0.04 mg/kg for rats compared to other dioxin congeners with a lethal dose up to 100 mg/kg (11). 2,3,7,8-TCDD activates the aryl hydrocarbon receptor (AhR) in humans that is involved in the regulation of biological responses to many aromatic hydrocarbons. Several adverse health effects associated with exposure to dioxins, especially to 2,3,7,8-TCDD, including tumor promotion, stomach cancer, toxicity to the skin, immunological, neurological, and developmental effects and adaptive responses such as the induction of drug metabolizing enzymes (12-16). When the receptor binds to dioxins and dioxin like compounds, it forms a ligand-receptor complex that then signals the activation of a number of genes.

Figure 1. Examples of dioxin like compounds

(A) Dibenzofuran, (B) Dibenzo-p-dioxin, and (C) 2,3,7,8-Tetrachlorodibenzo-p-dioxin.
coding for enzymes and other macromolecules responsible for numerous biological effects in the body (12, 17, 18).

As chlorinated dioxins became problematic environmental pollutants, thermal treatment was used as an efficient process for destroying dioxins and their chlorinated congeners along with many other physicochemical treatments (19). Economically, these processes are costly and not feasible to treat contaminated soils and sediments. Alternatively, biological treatments have been studied extensively by using bacterial strains to destroy these organic compounds due to their low cost and their small impact on the environment. Additionally, bacteria have important roles in the global carbon cycle due to their activity in degradation and mineralization of many aromatic compounds in natural environments and have the capability to completely degrade these compounds rather than transforming them to dead end intermediates (20-27).

**Degradation of dioxins by bacteria**

A large number of microorganisms have the capability to completely degrade organic pollutants and use these compounds as the sole source of carbon and energy for growth. Aerobic biodegradation includes key enzymatic reactions controlled by oxygenases which incorporate molecular oxygen (O$_2$) into an organic substrate either for activation or cleavage of the aromatic ring (28, 29). Activation of the aromatic ring is facilitated by enzymatic incorporation of oxygen by non-heme iron dependent dioxygenases that form dihydroxylated intermediates (30) or by flavin dependent monooxygenases that form singly hydroxylated intermediates (31). Hydroxylated intermediates (catecholic compounds) are cleaved by a ring cleavage dioxygenase which will be either an intradiol or extradiol dioxygenase to form the ring cleavage products that
will be hydrolyzed by a hydrolase and then will be channeled into central metabolic pathways (32, 33).

Increasing concern about accumulation of dioxins in the environment and the effects of these compounds have led to extensive studies to elucidate the mechanism in which microorganisms degrade dioxins and the fate of dioxins in the environment. Since that time, microbial degradation of biaryl ethers and dioxins were studied by using biphenyl, DBF, and DXN as models. However, studies on the bacterial degradation of DBF and DXN have been insufficient due to the difficulties in isolating bacteria capable to degrade dioxins especially the chlorinated congeners and for their high toxicities to humans. Most bacterial strains that have been characterized are only able to utilize DBF and they are not capable of growth on DXN as the sole source of carbon. Oxidation of DBF was first reported by using bacterial and fungal strains by formation of the cis- and trans-isomers of dihydroxy-dihydridibenzofuran as a dead-end intermediate (34). Oxidation of DXN and some of its chlorinated congeners were first reported by using Pseudomonas spp. and Beijerinckia spp. (now Sphingobium) that led to the formation of dead-end intermediates, cis-dihydrodiols (35, 36). The same intermediates were produced from DXN by a naphthalene degrading Pseudomonas strain (35). A biphenyl degrading strain Alcaligenes JB1 produced similar hydroxylated compounds from DBF and DXN and from chlorinated DXNs (37, 38). A carbazole degrading bacteria Pseudomonas spp. strain CA10 can cooxidize not only non-chlorinated DBF and DXN but also 2-CDBF, 2-CDXN, and 2,3-diCDXN to the corresponding chlorinated salicylic acid and chlorinated catechols (39). Terrabacter spp. DPO1361, formerly named Brevibacterium spp. DPO1361 and Sphingomonas spp. HH69, formerly named
*Pseudomonas* spp. HH69, were first characterized as DBF degraders through a novel angular dioxygenation mechanism and both can attack DXN and produce a 2,2',3-trihydroxybiphenyl ether intermediate (40-42). The DBF degrading bacterium *Terrabacter* sp. strain DBF63, formerly named *Staphylococcus auriculans* DBF63, can only cooxidize DXN into THBE and some chlorinated DXNs into the corresponding chlorinated catechols (25, 39). There are very few strains that have been isolated that have the capability to degrade both DBF and DXN. Among these strains, *Sphingomonas wittichii* strain RW1 is well characterized and its genome has been fully sequenced (43). It was isolated for its ability to degrade not only DBF but additionally utilizes DXN as a sole source of carbon. Compared to the other bacterial strains mentioned above, DXN is only oxidized and never led to a complete degradation (21).

**Degradation of dioxins by *Sphingomonas wittichii* RW1**

RW1 was isolated from the Elbe River for its ability to degrade both DBF and DXN as the sole source of carbon and energy for growth (21). It was initially identified as a *Sphingomonas* species and then was formally named *Sphingomonas wittichii* by Yabuuchi et al. (44). RW1 is a gram negative α-proteobacterium and grows under strictly aerobic conditions. In addition to non-chlorinated dioxins, it has been shown that RW1 has the capability to transform a number of chlorinated dioxins such as mono and dichlorodioxins (22, 45) and 1,2,3,4,7,8-hexachlorodibenz-\(p\)-dioxin (46). Its genome has been fully sequenced and consists of two mega plasmids, pSWIT01 (310 kb) and pSWIT02 (222 kb), and one chromosome (5,382 kb)(43, 47). RW1 was used to remediate polychlorinated DXN from soil (45) and from incinerator fly ash (48).
The DBF degradation pathway was first elucidated by an initial attack that could occur at the 4 and 4a carbon atoms in *Sphingomonas* sp. HH69 and *Brevibacterium* spp. (41, 42, 49). It has been proved that DBF degradation in HH69 is initiated by angular dioxygenation by observing accumulation of 2,2',3-THB from a spontaneous mutant disrupted in the meta cleavage dioxygenase (20). The same angular attack is present in RW1 (21) which is initiated by a multicomponent enzyme system named dibenzofuran 4,4a-dioxygenase (50). Similarly, the RW1 DXN degradation pathway was postulated to hydroxylate one of the two benzene rings by the same multicomponent enzyme, the angular dioxygenase, to yield 2,2',3-THBE after a spontaneous rearrangement. The hydroxylated ring is then subjected to meta cleavage based on the inhibition of the meta cleavage dioxygenase by 3-chlorocatechol and the observation that 2-(2-hydroxyphenoxy)-muconate, the product of the ortho cleavage dioxygenase, was never utilized or cooxidized by RW1 (21). The DBF, DXN, and biphenyl pathways are very similar and the only difference is that the second step in biphenyl degradation requires a dehydrogenase to form the dihydroxybiphenyl intermediate whereas in DBF and DXN pathways a dehydrogenase is not required and instead spontaneous rearomatization forms trihydroxybiphenyl and trihydroxybiphenylether (respectively) as a result of cleavage of the ether bond (21, 41, 49).

Transformation of DBF and DXN in RW1 is initiated by an angular dioxygenase complex, composed of four proteins, that leads to an extremely unstable hemiacetal intermediate which results in cleavage of the ether bond to form the more stable intermediates 2,2',3-THB and 2,2',3-THBE from DBF and DXN, respectively (21, 50, 51). In a subsequent step, a meta cleavage dioxygenase cleaves the hydroxylated aromatic
ring to yield the ring cleavage products, HOPDAs (52, 53). A hydrolase step hydrolyzes
the meta cleavage products 2-hydroxy-6-oxo-6-(2-hydroxyphenyl) hexa-2,4-dienoate and
2-hydroxy-5-(o-hydroxyphenoxycarbonyl)-2,4-pentadienoate from DBF and DXN,
respectively (51, 53, 54). The two yield products, salicylate and catechol, channel into the
central metabolic pathway (Figure 2).

It has been shown that all genes involved in DBF and DXN degradation in RW1 are not clustered together but rather are dispersed in the genome and located in several loci (55) and have been thought to be located on the smaller mega plasmid, pSWIT02 (50, 56-58). More related genes were found to be located on the chromosome with the presence of large numbers of oxygenases and hydrolases with a high degree of redundancy in the genome (43, 47, 59). The exact genes that are involved in the second and third enzymatic steps in dioxin degradation in RW1 remains unknown. In this study our goals are to identify the exact ring cleavage dioxygenases and hydrolases that are involved in each pathway.
Figure 2. Catabolic pathway for dibenzo-\(p\)-dioxin and dibenzofuran degradation by \textit{S. wittichii} RW1.
**Angular dioxygenation of dioxins in RW1**

DBF and DXN were chosen as a model for studying the degradation of biaryl ether compounds (41). A novel dioxygenation mechanism of DBF by *Brevibacterium* spp, and DBF and DXN by *Sphingomonas* HH69 has been suggested and described as an unusual angular dioxygenation (40, 41, 49). The same angular dioxygenation was found in RW1 and it has been shown that this dioxygenase system, named dibenzofuran 4,4a-dioxygenase, hydroxylates DBF and DXN by introducing two oxygen atoms at a pair of vicinyl carbon atoms and one of them hydroxylates the carbon atom adjacent to the ether bridge connecting the two aromatic rings (21, 50, 55).

This enzyme has been purified as a three component system belongs to the superfamily of Rieske non-heme iron dioxygenases that consists of two oxygenase subunits, a reductase, and a ferredoxin (50). The enzyme hydroxylates DBF and DXN at positions 4 and 4a to form an extremely unstable hemiacetal-like compounds that rearomatizes by a spontaneous loss of a phenolate group with the cleavage of the ether bridge to yield 2,2′,3-THB and 2,2′,3-THBE from DBF and DXN, respectively (50, 60).

Two isofunctional reductases were identified as components A1 and A2 that have molecular weight of about 44-KDa each (50). RedA2 was biochemically purified and identified as a class I cytochrome P-450-type (57). Both identified reductases, RedA1 and RedA2, have similar activity with ability to transfer electrons from NADH to an electron acceptor, a ferredoxin, and both contain a flavin adenine dinucleotide cofactor type. Interestingly, this type of reductase differs from other known reductases associated with aromatic electron transport systems by lacking an iron-sulfur cluster. Additionally, there
was no significant match with their N-terminal sequence comparing with other known reductases (50, 55, 57).

The ferredoxin, 12-kDa component B, was identified as a part of the multicomponent dioxygenase system and transports the electrons from the reductase to the terminal oxygenase (50). This ferredoxin named Fdx1 and characterized as a putidaredoxin-type 2Fe-2S ferredoxin (a "plant type" ferredoxin) has been genetically shown to not be linked directly with the genes for the two dioxygenase subunits (61). A similar gene to \textit{fdx1} was located downstream of the genes for the two dioxygenase subunits located on pSWIT02 plasmid and named \textit{fdx3} (55, 62). It was biochemically and genetically characterized as a substitute ferredoxin for Fdx1 that can serve as an electron donor for the dioxin dioxygenase (58).

Multiple ring hydroxylating dioxygenase subunits were identified in RW1, component C (55). Two heterodimeric terminal oxygenases DxnA1 and DxnA2 with large 45-KDa α and with small 23-KDa β subunits were purified and overexpressed in \textit{E. coli} beside two different electron transfer systems, class IIA-type from \textit{Burkholderia xenovorans} LB400 and \textit{E. coli} class IIB-type electron chain (55). Their results showed no activity at all when DBF and DXN were used as a substrate in the presence of these two electron transfer systems. Full activity was obtained only when cells containing the two dioxygenase subunits coexpressed with the cognate electron transfer system containing \textit{fdx1} and \textit{redA2} from RW1.

Due to the unusual properties of the reductase and the ferredoxin mentioned above, this angular dioxygenase was classified as a class IIA dioxygenase system (50). Other dioxygenases that oxidize DBF and DXN in an angular dioxygenation have also
been described from other bacterial strains such as *Sphingomonas* sp. HH69 (63), the carbazole 1,9a-dioxygenase from *Pseudomonas resinovorans* CA10 (64), the gram positive DBF degrading bacteria *Terrabacter* sp. strain DBF63 (65) and *Terrabacter* sp. strain YK3 (66).

Most aromatic compound dioxygenases that have been studied have a genetic organization identified by clustering of not only the genes comprising the initial dioxygenase but also for the entire pathway or at least they are transcribed from a single transcriptional unit (30). This is completely different from what has been found in the initial dioxygenase for DBF and DXN in RW1, as it has been shown by (55) that the genes coding the electron chain ferredoxin, \( fdx1 \), and the reductase are not clustered with the two-dioxygenase subunits \( dxnA1A2 \) but rather are located on different segments of DNA and transcribed separately. Additionally, more genes coding for the ferredoxin and the reductase were located on the chromosome that might involve and have function in DBF and DXN degradation. Scattering the genes not only on the mega plasmid but also on the chromosome is not really understood and might indicate involvement of these genes in other catabolic pathways as RW1 has large number of oxygenases (43, 47, 59).

**Meta cleavage dioxygenases in RW1**

Ring cleavage dioxygenases are of interest due to their importance in degradation of many environmental pollutants and their role in limiting the specificity of the pathway especially for chlorinated aromatic compounds (67, 68). Attention has increased in understanding the role of ring cleavage dioxygenases and many techniques have been used to alter their specificity to increase their activity for aromatic compound degradation
Extradiol dioxygenases were first recognized among other ring cleavage dioxygenases and identified as a non heme iron containing enzyme (73).

The second enzymatic step in dioxin degradation was identified by involvement of an extradiol dioxygenase that cleaves the aromatic ring substrate between one hydroxylated carbon atom and adjacent non hydroxylated carbon atom and usually they are a multimer of a single type of subunit that contains ferrous iron (32). Their involvement in many aromatic catabolic pathways assigned this type of enzyme as a major determinant of substrate specificity of many pathways such as the toluene pathway (74, 75) and degradation of some chlorinated biphenyls (76).

The ring cleavage enzyme of dioxin degradation was first studied in RW1 strain by Happe et al. (52) and its function was identified as a meta cleavage of 2,2',3-THB and 2,2',3-THBE from DBF and DXN. This 32-KDa enzyme was named DbfB and has been genetically and biochemically characterized as a monomeric structural protein, unlike most other known extradiol dioxygenases that have an oligomeric structure. The enzyme was hyper expressed in *E coli* and purified and its kinetics showed its capability to cleave 2,2',3-THB and 2,3-DHB from DBF and biphenyl, respectively. Whereas, its activity against 2,2',3-THBE, 3-methylcatechol, 4-methylcatechol, catechol and 3,4-DHB is much lower. In fact, they could not measure its activity against 2,2',3-THBE accurately. Oxygen consumption experiments showed that the substrate 2,2',3-THBE had only 15% of the activity as for 2,3-DHB. Also, *dbfB* was shown to have the highest specificity for 2-chlorohydroxybiphenyl compared with BphC from *Burkholderia* spp. strain LB400 and *Rhodococcus globerulus* P6 (69). According to its substrate specificity and the sequence similarity with other extradiol dioxygenases, DbfB is classified into the subfamily of
extradiol dioxygenases with high activity towards bicyclic compounds (77). Interestingly, \textit{dbfB} is not clustered with the genes for the oxygenase component but rather is located 4.5 kb upstream, oriented in the opposite direction (55), and constitutively expressed (52, 53).

The genome sequence of RW1 indicates that it has about 34 extradiol dioxygenases (43, 59). The high number of dioxygenases in RW1 reflects the versatility of metabolic pathways that might be present in RW1. Only one ring cleavage dioxygenase, DbfB, has been genetically and biochemically characterized and its affinity to 2,2',3-THBE is very low (52) that gives an indication that another ring cleavage dioxygenase might be involved in the dioxin pathway. A proteomic study showed upregulation of another ring cleavage enzyme located on the chromosome encoded by SWIT3046 when DBF was used as a sole source of carbon compared with acetate and they could not detect \textit{dbfB} expression in this study (78). Another proteomic study has suggested involvement of SWIT3046 in dioxin degradation and assuming its function is more important in transformation of 2,2',3-THBE as its detection was higher in response to dibenzo-\textit{p}-dioxin than DBF as a substrate (59).

**Hydrolases in RW1**

All meta cleavage products are channeled into two main pathways; the hydrolytic pathway or the dehydrogenase pathway (79). The latter pathway requires the cells to synthesize three enzymes including isomerases and decarboxylases; whereas, only one enzyme is required for the hydrolytic pathway. The existence of two pathways is assumed to play an important role in catalyzing different substrates in an energy favorable fashion (80).
The third enzymatic step in dioxin degradation was identified by involvement of two isofunctional hydrolases named H1 (DxnB1) and H2 (DxnB2) (53, 55). These enzymes are able to catalyze C-C bond cleavage of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate to form salicylic acid and benzoic acid, respectively (53). They were identified as monomeric proteins and they share 50% identity at the N-terminus and both enzymes are expressed constitutively in RW1 (53). In addition to salicylate, the C5 moiety produced by the hydrolase enzyme was assumed to be 2-oxo-4-pentenoate (53) as this intermediate was detected from DBF degradation by *Brevibacterium* spp. strain DPO 1361 (81). DxnB2 was shown to have higher specificity towards 3-chloro-HOPDAs and 3,9,11-trichloro-HOPDA compared with the two well-known hydrolases BphD from *B. xenovorans* LB400 and *Rhodococcus globerulus* P6 (54).

These types of hydrolases belong to the alpha/beta hydrolase fold enzymes. They are structurally related, and their characteristic properties are much conserved among all known meta cleavage hydrolases (82, 83). They have a catalytic triad consisting of nucleophile-acid-histidine and the nucleophile for all known ring cleavage product hydrolases is always a serine residue (84).

Hydrolases are a key determinant of the bacterial ability to degrade specific intermediates from various aromatic compounds (85-87). For instance, BphD from *Burkholderia xenovorans* LB400 is a key determinant in the catabolic pathway of polychlorinated biphenyls (87). Also, it has been shown that aromatic hydrolases are a critical step in dibenzofuran degradation due to the spontaneous formation of the dead end intermediate 3(chroman-4-on-2-yl)pyruvate that requires a hydrolase with a high
affinity to the substrate to overcome the spontaneous formation of this intermediate (20, 21, 81).

Ring cleavage product hydrolase reactions are considered as a rare enzymatic reaction in chemistry and biochemistry due to the absence of any cofactor in the reaction (88, 89). The two hydrolases identified in RW1 are located at different loci and only \( dxnB1 \) is clustered with the genes for the oxygenase subunits, \( dxnA1A2 \), located on pSWIT02. \( dxnB2 \) is located on the chromosome (55) upstream of a putative salicylate hydroxylase, SWIT3056 (90).

The involvement of hydrolases in DBF and DXN degradation in RW1 is not clear and whether the two hydrolases, \( dxnB1 \) and \( dxnB2 \), hydrolyze the ring cleavage products from the two substrates or from only one substrate is unknown. Additionally, a proteomic study showed upregulation of another hydrolase in dioxin degradation in RW1. Its detection was highest in response to 2-chlorodibenzo-\( p \)-dioxin than dibenzo-\( p \)-dioxin and dibenzofuran degradation (59). This hydrolase, encoded by SWIT0910, is located on the chromosome and 3.17 Mb upstream of \( dxnB2 \) and oriented in same direction.

Despite the presence of a large number of hydrolases (about 40) in RW1 (43, 59), there are only a few studies which characterize selected RW1 hydrolases and their roles in the various aromatic degradation pathways are not completely known. The fact that the formation of salicylate from DBF requires a hydrolase that cleaves a C-C bond and the formation of catechol from DXN requires a hydrolase that cleaves a C-O bond suggests a multifunctional hydrolase or multiple hydrolases involved in the two pathways (21, 51). A central (proven) hypothesis of our research is that multiple hydrolases are required for the degradation of the structurally related compounds DBF and DXN.
Chapter 2: Growth of *Sphingomonas wittichii* RW1 on Dibenzo-\(p\)-dioxin

Requires a Chromosomally Encoded Ring Cleavage Dioxygenase

**Introduction**

*Sphingomonas wittichii* RW1 was isolated from the Elbe River for the ability to degrade dibenzo-\(p\)-dioxin (DXN) and dibenzofuran (DBF)(21). The proposed upper catabolic pathway is initiated by an angular dioxygenase, dibenzofuran 4,4a-dioxygenase (50) that catalyzes the conversion of DXN and DBF to 2,2',3-trihydroxybiphenylether (THBE) and 2,2',3-trihydroxybiphenyl (THB) respectively. The latter compounds are then cleaved by a meta ring cleavage dioxygenase (52) and a hydrolase (53) converts the meta cleavage products to catechol and salicylate respectively which then enter central aromatic metabolic pathways in the cell. The *S. wittichii* RW1 genome sequence is known (43) and many of the genes involved in the DXN/DBF catabolic pathway have been identified (55, 58) and localized to the RW1 pSWIT02 plasmid. While the RW1 enzymes involved in the DXN and DBF catabolic pathways are thought to be produced constitutively (21) there have been multiple transcriptomic and proteomic studies to identify differentially expressed RW1 genes under different growth conditions (59, 78, 90, 91).

Unique to RW1 is the ability to degrade both DXN and DBF. To our knowledge, only two other bacterial strains are reported to grow on DXN as the sole source of carbon (92, 93). Several bacteria are known to grow on DBF but to only co-oxidize DXN (63, 65, 94). This is a unique conundrum: if the structurally related compounds DXN and DBF are degraded by similar catabolic pathways then why is it easy to find strains that grow on DBF and so hard to find strains that grow on DXN and why don't strains that
grow on DBF also grow on DXN? It is prevailing wisdom that the genes for the DBF and DXN upper catabolic pathways are contained on the RW1 pSWIT02 plasmid (43, 55) but pSWIT02-like plasmids are found in other bacteria and these strains grow on DBF but not DXN (63, 95, 96).

Purification and characterization of the RW1 dibenzofuran 4,4a-dioxygenase (50) indicates that this ring hydroxylating dioxygenase catalyzes the transformation of DXN at 84% the rate at which it transforms DBF. Expression of the RW1 dxnA1A2 oxygenase genes in *E. coli* along with the cognate ferredoxin and reductase electron transfer system (55, 58) confirmed that a single enzyme has both DXN and DBF ring hydroxylating activities. Thus a single dioxygenase enzyme initiates the degradation of both DXN and DBF in RW1 and it is not surprising that other bacteria that grow on DBF can co-oxidize DXN. The second enzyme in the RW1 DBF catabolic pathway, 2,2’,3-trihydroxybiphenyl 1,2-dioxygenase (THB 1,2-dioxygenase) has also been extensively studied (52). This meta ring cleavage enzyme is encoded by the *dhfB* gene located 4.5 kb away from the *dxnA1A2Bfdx3* operon on pSWIT02 in RW1 (43, 52). Interestingly, even though DbfB has a $k_m$ for 2,3-dihydroxybiphenyl and THB in the micromolar range and a $k_m$ for catechol and 3-(or 4-)methylcatechol in the millimolar range the activity against THBE as a substrate was too low to generate kinetic parameters (52). This means that DbfB would function poorly, if at all, in the DXN catabolic pathway.

Therefore, it is our hypothesis that the pSWIT02 encoded DbfB meta ring cleavage enzyme functions only in the DBF catabolic pathway and not in the DXN catabolic pathway. We used genomic and transcriptomic guided approaches to identify a second meta ring cleavage enzyme involved in the upper pathway for DBF and DXN
degradation. The role of both DbfB and the newly discovered meta ring cleavage enzyme in DBF and DXN metabolism was verified through gene knockouts and physiological analysis of the resulting mutant strains.

**Materials and Methods:**

**Bacterial strains, plasmids, media and growth conditions.** *Sphingomonas wittichii* RW1 is the wild type strain capable of utilizing dibenzofuran and dibenzo-\(p\)-dioxin as the sole carbon and energy source (21). *E. coli* DH5\(\alpha\) was the recipient strain in all cloning experiments. The pGEM-T easy vector (Promega, Madison, WI) was used to clone PCR products. pRK415 (97) was used to construct clones for gene knockout and for complementation experiments. Mineral salts basal medium [MSB] (98) was used for carbon source and metabolite accumulation studies. When needed, L-phenylalanine was added to MSB at 10 mM final concentration. DBF and DXN were dissolved in acetone in a sterile flask to a calculated final concentration of 3 mM and the flasks left in a fume hood for 5 to 6 hours to allow for complete evaporation of the acetone. After addition of MSB medium the flasks were sonicated in a Branson 1800 water bath sonicator for 5 minutes to disperse the substrate crystals throughout the medium. DBF and DXN crystals were added on the lid of the MSB agar petri dish for growth tests as the sole carbon source. Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, MO) was added at 2 mg/ml MSB broth when needed. LB agar and LB broth were used as complete media. RW1 wild type and all mutants were grown aerobically at 30\(^\circ\)C and *E. coli* strains were aerobically cultured in LB medium at 37\(^\circ\)C. Ampicillin, gentamicin, kanamycin, and tetracycline were added to the medium when needed at 100, 15, 50, and 15 \(\mu\)g/ml, respectively.
**DNA Techniques.** Total genomic DNA from RW1 and RW1-derived strains was prepared using the Qiagen (Germantown, MD) Ultra Clean Microbial Kit and plasmid DNA was purified using the Macherey-Nagel (Bethlehem, PA) NucleoSpin Plasmid Kit following the manufacturers’ instructions. Transformation of plasmid DNA into competent *E. coli* strains was performed by the calcium chloride-glycerol transformation procedure (99). PCR products and restriction fragments were purified using the GeneClean III Kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions. Restriction enzymes and ligations of DNA samples were performed as recommended by the supplier (New England Biolabs, Ipswich, MA). All PCR amplifications were performed with the NEB Phusion High-Fidelity kit when the genes were needed for downstream applications and with NEB Taq when used for mapping and screening purposes following instructions from the supplier. All PCR generated fragments used for downstream applications were sequenced to verify that no base changes were introduced. DNA sequencing was conducted by Genewiz (South Plainfield, NJ).

**Construction of ring-cleavage dioxygenase knockout mutants and complementation.** Gene inactivation was achieved by disrupting the gene with an antibiotic cassette and was introduced into the chromosome by homologous recombination. The *dbhB* gene was amplified by PCR using RW1 chromosomal DNA with flanking regions 0.59 kb upstream of the gene start codon and 0.5 kb downstream of the gene stop codon using the primers GGGGAATTCCAaatcctcgtgacacagggc and CCAAGCTTgccggtacccctctcgtcgtgc containing *Eco*RI and *Hind*III restriction sites, respectively. The resulting 1.98 kb DNA fragment was gel purified, digested with *Eco*RI
and HindIII and ligated into similarly digested pRK415 to form pRK_dbfBKO and then was disrupted with a kanamycin resistance cassette that was PCR amplified using p34S-Km3 plasmid as a template (100) with the primers CCCCTCGAGcgagctcaaagccacgttgtctc and CCCTCGAGcgggtaccgagctcttagaaaaactc containing XhoI restriction site for both ends, digested with XhoI and ligated into similarly digested pRK_dbfBKO to form pRK_dbfBKO-km. The final construct was transformed into DH5α and sequenced.

SWIT3046 was amplified by PCR with a flanking region 0.9 kb upstream of the gene start codon and 0.35 kb downstream of the gene stop codon with the primers GGAAGCTTcagatggggagcaacaacatggc and GGTCTAGAgtgcgtcgaccaggagtagatg containing HindIII and XbaI restriction sites, respectively. The resulting 2.1 kb DNA fragment was purified and digested with HindIII and XbaI and ligated into similarly digested pRK415 to form pRK_3046KO. A gentamicin resistance cassette was used to disrupt SWIT3046 in the unique XhoI site after digestion of p34S-Gm (100) with SalI and ligated into XhoI digested pRK_3046KO (SalI and XhoI have compatible ends). The resulting construct pRK_3046KO-gm was transformed into DH5α and sequenced.

The two constructs pRK_dbfBKO-km and pRK_3046KO-gm were individually transferred into RW1 by triparental mating using the helper E. coli pRK2013 with selection on MSB supplemented with phenylalanine and tetracycline. The transconjugants were cultured in 5ml LB broth and transferred into new 5ml LB broth every day allowing for the double recombination event to occur. To screen for recombination, colonies were tested for Km (dbfB knockout) or Gm (SWIT3046 knockout) resistance and Tc sensitivity on LB agar. The presence or absence of the
plasmid constructs and the occurrence of the desired double recombination event were verified by PCR using the above mentioned primers for each construct.

Complementation of the mutants was performed by cloning dbfB and SWIT3046 into pRK415 downstream of the constitutive dxnA1 promoter. For dbfB a 0.44 kb fragment of the dxnA1 promoter region was PCR amplified using primer GGGAAGCTTGCCCTGTCTCCGAGATGCTCTG (containing a HindIII site) and primer ctccaaACCTGCAGCAACCTCTCCTGCAT (containing a PstI site and containing an overlap sequence with the dbfB upstream fragment primer). A 0.94 kb region of dbfB was PCR amplified using the primer GCCTGCAGGGtttgagcaaaaggagagt (containing a PstI site and containing an overlap sequence with the dxn promoter downstream fragment primer) and TCTAGAgtgtgccttaattaattcaatgcgc (containing a XbaI site). The two fragments were purified and ligated by PCR overlap extension using the following protocol: the two PCR fragments were mixed in one PCR reaction at a 1:1 ratio using the Phusion HF PCR kit without adding any primer under the following conditions: denaturation at 98°C for 10 sec, anneal at 72°C for 40 sec, and extension at 72°C for 1 minute for 10 cycles then primer GGGAAGCTTGCCCTGTCTCCGAGATGCTCTG and primer TCTAGAgtgtgccttaattaattcaatgcgc and the reaction was returned to the PCR machine for another 25 cycles under the same conditions. The resulting 1.38 kb fragment was purified from an agarose gel, digested with HindIII and XbaI and cloned into similarly digested pRK415 to yield pRK_PdbfB, transformed into DH5α, and sequenced to verify.

For the second expression clone a 0.97 fragment containing SWIT3046 was PCR amplified using primer GGAAGCCTTcgcgtgcgtttctccatcatg (containing a HindIII site)
and primer GGTCTAGAgcaggtctccgacccggtg (containing a XbaI site). The resulting fragment was purified from an agarose gel and cloned into pGEM-T to yield pGEMT_3046 and sequenced to verify. The *dxnA1* promoter was PCR amplified using primer CTGCAGgcctgtctccgagatgtct (containing a PstI site) and AAGCTTgcaacctctctgcactcct (containing a HindIII site). The resulting 0.44 kb fragment was purified from an agarose gel and digested with PstI and HindIII. pGEMT_3046 was digested with HindIII and XbaI and purified from an agarose gel. The two purified fragments, 0.44kb and 0.97 kb, were cloned into pRK415 digested with PstI and XbaI by a three-way ligation to yield pRK_P3046.

The final constructs pRK_PdbfB and pRK_P3046 were transformed into DH5α and transferred into the appropriate mutant strain by triparental mating with selection on MSB phenylalanine and tetracycline. The presence of the constructs in the RW1 mutants were verified by PCR.

**Growth curves.** RW1, RW1ΔdbfB, and RW1Δ3046 were incubated overnight on LB agar with the appropriate antibiotic at 30°C. LB broth (50 ml) was inoculated with a loopful of cells and incubated over night at 30°C in a rotary shaker (180 rpm). MSB broth (50 ml) with phenylalanine was inoculated from the LB broth culture with a starting OD at 600nm of 0.05 and incubated at 30°C in a rotary shaker (180 rpm). The strains were grown in MSB phenylalanine twice and when the optical density of the cells reached 2.0 in the second culture triplicate MSB broth cultures containing either 3 mM DBF or 3 mM DXN were inoculated from each strain and OD readings taken at specific times. All growth curves were plotted using the Prism software (GraphPad, San Diego, CA).
**Gene replacement constructs.** Since RW1ΔdbfB grows slowly on DBF, gene replacement was carried out to determine if it is a promoter problem by swapping SWIT3046 from the chromosome into pSWIT02 in place of dbfB and swapping dbfB from pSWIT02 into the chromosome in place of SWIT3046. A 1.05 kb region upstream of the dbfB start codon including the dbfB ribosomal binding site was amplified by PCR using the primers GGAAGCTTcgttagcctctcacgagtg (containing a HindIII restriction site) and gatttcagacatgacgataactctcctttgc (containing overlap sequence with SWIT3046). SWIT3046 was PCR amplified from the start codon without its ribosomal binding site using the primers gagttatcgtcatgtctgaaatctcgagcc (containing overlap sequence with the dbfB upstream fragment primer) and gtgtatggcCTCTAGAGtaatgcgcgtgcgcgtg (containing overlap sequence with the dbfB downstream fragment and an XbaI restriction site for adding the antibiotic cassette). A 1.05 kb region downstream of dbfB was PCR amplified using the primers cgccgctgtgaCTCTAGAggcatacacctaacattgc (containing overlap sequence with the primer for the end of SWIT3046 and the XbaI restriction site) and CCGGATCCcacctcaagcgcatcctg (containing a BamHI restriction site). All three fragments were ligated by PCR overlap extension using the following protocol: the three PCR fragments were purified and mixed in one PCR reaction at a 1:1:1 ratio using the Phusion HF PCR kit without adding any primer under the following conditions: denaturation at 98°C for 10 sec, anneal at 72°C for 40 sec, and extension at 72°C for 2 minutes for 10 cycles then the following primers were added GGAAGCTTcgttagcctctcaagtg (the forward primer from upstream fragment-HindIII) and CCGGATCCcacctcaagcgcatcctg (the reverse primer for downstream fragment-BamHI) and the reaction was returned to the PCR machine for another 25 cycles under
the same conditions. The resulting 3 kb fragment was purified from an agarose gel and cloned into pGEM-T to yield pGEMT_fB3046, transformed into DH5α, and sequenced. pGEM_fB3046 was digested with HindIII and BamHI and ligated into the similarly digested pRK415 to make pRK_fB3046. A kanamycin antibiotic cassette was digested from p34S-Km3 with XbaI and ligated into similarly digested pRK_fB3046. The final construct pRK_fB3046-km was transferred into RW1 and RWΔ3046 by triparental mating to obtain the RW1ΔdbfB/3046 (dbfB replaced by SWIT3046 = two copies of SWIT3046) and RW1Δ3046ΔdbfB/3046 (SWIT3046 knockout with dbfB replaced by SWIT3046 = one copy of SWIT3046).

Using a similar approach SWIT3046 was replaced by dbfB. A 1.1 kb upstream region just before the start codon of SWIT3046 and including the ribosome binding site, was PCR amplified using the primers GGAAGCTTaccggcttccgggctacacgctgct (containing a HindIII site) and ttactgacatcggaacacctcatgatcaggatcgtc (containing overlap sequence with the beginning of dbfB). A 0.88 kb region of dbfB was amplified starting from the start codon without its ribosome binding site using the primers gaggtggtccgtggtcataaaaaacttggc (containing overlap sequence with the end of the upstream fragment) and CGCGGGATCCTCAATGCGCCGGCAACTG (containing a BamHI site). A 1.1 kb downstream region of SWIT3046 was PCR amplified using the primers ggcgcattgagaGGATCCcgcggccacccggctcag (containing an overlap sequence with the end of dbfB and a BamHI site for adding an antibiotic cassette) and CCGGTACCgagcagctcgccggccgct (containing a KpnI site). The three fragments were ligated by PCR overlap extension using the same conditions mentioned above and after 10 cycles the primers GGAAGCTTaccggcttccgggctacacgctgct (forward of upstream
fragment with \textit{HindIII}) and CCGGTACCgagcagctccgcgccggcct (reverse of downstream fragment-\textit{KpnI}) and the reaction was returned to the PCR machine for another 25 cycles. The resulting 3 kb DNA fragment was gel purified and cloned into pGEM-T to make pGEMT\_f3046B, transformed into DH5α, and sequenced. The 3 kb \textit{HindIII} and \textit{KpnI} fragment was removed from pGEMT\_f3046B and ligated into similarly digested pRK415 to make pRK\_f3046B. A kanamycin cassette from p34S-Km3 was digested with \textit{BamHI} and ligated into similarly digested pRK\_f3046B. The final construct pRK\_f3046B-km was transferred by triparental mating using pRK2013 into RW1 and RW1ΔdbfB to construct the knockout strains RW1Δ3046/dbfB (SWIT3046 replaced by \textit{dbfB} = two copies of \textit{dbfB}) and RW1ΔdbfBΔ3046/dbfB (\textit{dbfB} knockout with SWIT3046 replaced by \textit{dbfB} = one copy of \textit{dbfB}).

\textbf{Results}

\textbf{Role of DbfB in DBF and DXN degradation by RW1}

\textit{S. wittichii} RW1 degrades DBF and DXN through similar pathways. Crucial to these pathways is cleavage of the first aromatic ring. The RW1 upper pathway meta ring cleavage enzyme encoded by \textit{dbfB} has been well studied \cite{52}. We were intrigued by the fact that the enzyme has high activity toward the hydroxylated biphenyl compounds 2,3-dihydroxybiphenyl and THB (biphenyl and DBF pathway metabolites) but has low activity toward the hydroxylated biphenvylether compound THBE (DXN pathway intermediate). The only difference between these two compounds is the oxygen atom joining the two rings in THBE. As described in the Materials and Methods we knocked out \textit{dbfB} (SWIT4902) resulting in the strain RW1ΔdbfB. This strain does not grow on
DBF and a brown colored compound identified as THB appears in the medium. The strain is thus effectively blocked in the upper pathway ring cleavage step. Interestingly, RW1ΔdbfB grows normally on DXN and does not accumulate any noticeable compounds in the culture medium. The inability of RW1ΔdbfB to grow on DBF could be due either to a complete blockage in the catabolic pathway or a partial blockage and the toxic effects of the accumulating THB. We therefore analyzed growth of RW1ΔdbfB on both DBF and DXN with Amberlite XAD7HP resin added to adsorb and sequester any THB formed. Growth curves of the wild type RW1 on DXN and DBF with and without the added resin are nearly identical (data not shown). Interestingly, growth curves of RW1ΔdbfB with DBF as the substrate show that deletion of this gene simply slows the growth on DBF (Figure 3). Surprisingly, growth curves of RW1 and RW1ΔdbfB with DXN as the sole carbon source are nearly identical (Figure 4) showing that deletion of the *dbfB* encoded upper pathway meta cleavage enzyme is not needed for metabolism of DXN. These data mean that there must be a second ring cleavage enzyme in RW1 that functions in both the DXN and DBF catabolic pathways.

**Identification of a second ring cleavage enzyme involved in DXN and DBF degradation by RW1**

We used a genomic and transcriptomic approach to identify a second ring cleavage enzyme involved in DXN and DBF degradation by RW1. Two transcriptomic and two proteomic studies have been performed on RW1 (59, 78, 90, 91). Studies such as these often report gene expression as a ratio between induced and uninduced cultures but early published work showed that many of the RW1 enzymes involved in DXN and DBF metabolism are constitutively produced (21). By our count there are 14 different
meta ring cleavage enzymes encoded in the genome of RW1 (Figure 5). We thus examined the raw RNA-seq counts in the Chai et al. transcriptomic study (91) for the genes encoding these 14 different ring cleavage enzymes following growth of RW1 on succinate, DBF, and DXN. Of the 14 different genes examined we found that SWIT4902 (dbfB) on the pSWIT02 plasmid and SWIT3046 on the chromosome are expressed at high levels on all three growth substrates (Table 1). We thus knocked out the SWIT3046 gene as described in the Materials and Methods resulting in strain RW1Δ3046. This knockout strain accumulates THBE from DXN and did not grow on DXN as the sole carbon source even with the added resin (Figure 4). On the other hand, RW1Δ3046 grew normally on DBF as the sole carbon source (Figure 3). In order to confirm and extend these results the double knockout strain RW1ΔdbfBΔ3046 was constructed. As expected, this strain did not grow on either DXN or DBF (Figure 3 and Figure 4). These data prove that chromosomally encoded SWIT3046 is absolutely required for RW1 growth on DXN. The data also prove that plasmid encoded SWIT4902 (DbfB) is the best enzyme for RW1 growth on DBF and that SWIT3046 can partially replace SWIT4902 when the latter gene is knocked out.

**Complementation and gene replacement**

In order to prove that the growth experiments described above are due to the specific gene knockout and not due to a polar effect on downstream genes complementation experiments were performed. Each of the knockout strains was complemented by the analogous gene (either SWIT4902/dbfB or SWIT3046) cloned into pRK415 under control of the constitutive dxn promoter. Complementation of each knockout mutant restored wild type growth (Figure 6 and Figure 7) indicating that the
growth phenotypes described above are directly due to loss of either SWIT4902/dbfB or SWIT3046. Interestingly, pRK_P3046 completely restored wild type growth of the RW1ΔdbfBΔ3046 double knockout on DBF. In the single knockout RW1ΔdbfB the wild type SWIT3046 in the chromosome only partially restored growth. We hypothesize that restoration of wild type growth by pRK_P3046 is due either to more than one copy of SWIT3046 (pRK415 is a low copy number vector with 4 to 6 copies per cell) and/or due to expression of SWIT3046 from the dxn promoter cloned in front of the SWIT3046 gene in pRK415.

In order to determine if position in the genome (different promoter) effects the enzymatic activity of SWIT3046 the gene encoding this enzyme was substituted for the dbfB gene. Two strains were constructed as outlined in Figure 8. Strain RW1ΔdbfB/3046 has gene SWIT3046 substituted for dbfB and thus has two copies of SWIT3046. Strain RW1Δ3046ΔdbfB/3046 has gene SWIT3046 substituted for dbfB, the wild type SWIT3046 gene is knocked out, and thus the strain has only one copy of SWIT3046 (moved from the chromosome to pSWIT02). These two strains grew on DXN and DBF at the same rate and extent as the wild type strain RW1 (Figure 10 and Figure 11). These data thus show that SWIT3046 in its wild type chromosome location can only partially complement a dbfB knockout mutation but that SWIT3046 in the dbfB location fully complements a dbfB mutation. This is most likely due to the dbfB promoter being stronger than the SWIT3046 promoter and is also borne out by the RNA-seq data (91) where raw counts of dbfB are 3.2 times higher during growth on DXN and 21.8 times higher during growth on DBF compared to SWIT3046 (Table 1).
In order to extend and confirm these results the reverse experiment of substituting SWIT3046 with *dbfB* was performed. Two strains were constructed as described in Figure 9. Strain RW1Δ3046/dbfB has gene *dbfB* substituted for SWIT3046 and thus has two copies of *dbfB*. Strain RW1ΔdbfBΔ3046/dbfB has gene *dbfB* substituted for SWIT3046, the wild type *dbfB* is knocked out, and thus the strain has only one copy of *dbfB* (moved from pSWIT02 to the chromosome). As expected, neither strain grew on DXN (Figure 11) confirming the importance of SWIT3046 in DXN degradation. Two copies of *dbfB* did not increase growth on DBF compared to the wild type RW1 (Figure 10) indicating that other factors control the maximal rate of growth on this compound. However, a single copy of *dbfB* in the chromosome and linked to the SWIT3046 promoter grew slower than the wild type strain on DBF again demonstrating that the SWIT3046 promoter is not as strong as the *dbfB* promoter (Figure 10). Taken together these data demonstrate that both enzyme specificity and quantity of the enzyme produced (promoter strength) governs the rate and ability of RW1 to grow on either DXN or DBF.

**Discussion**

*S. wittichii* RW1 is one of only a very few organisms that can grow on DXN as the sole source of carbon and energy. Due to the environmental importance of DXN degradation, RW1 has been the subject of intense studies ranging from genetics and biochemistry to field applications. Two important questions remain to be answered: Exactly which enzymes are involved in the DXN and DBF catabolic pathways? and How did this organism evolve? The RW1 DXN and DBF catabolic pathway enzymes have been purified and characterized; genes have been cloned and heterologously expressed; and transcriptomic, proteomic, and Tn-Seq information has been generated. To our
knowledge the work presented here represents the first time that knockout mutagenesis and physiological characterization has been used to identify genes and enzymes involved in RW1 catabolic pathways.

It has been known for over 25 years that the RW1 upper pathway ring cleavage enzyme DbfB has strong activity toward the DBF metabolite THB and weak activity toward the DXN metabolite THBE (52). The work presented here confirms that conclusion since knockout of dbfB results in a strain that grows weakly on DBF but normally on DXN. Using a genomic and transcriptomic guided approach we identified a second ring cleavage enzyme, SWIT3046, which is absolutely required for RW1 growth on DXN. SWIT3046 can partially replace DbfB explaining why dbfB knockouts still grow on DBF. The involvement of SWIT3046 and dbfB in DBF and DXN metabolism is summarized by the metabolic map in Figure 12. It is important to note that understanding the role of gene expression levels is crucial in determining the role of enzymes in catabolic pathways. Positioning SWIT3046 downstream of a stronger promoter increased the ability of the enzyme to complement a dbfB knockout mutation, increasing the level of complementation from partial to full as exhibited by DBF growth curves of the various RW1 constructs. If SWIT3046 was not constitutively expressed and was induced by DXN then the ability to partially replace dbfB would not have been naturally detected. Thus, in wild type strains not only the enzyme characteristics are important (i.e. Vmax and Km for a particular substrate) but also the level of gene expression is important in determining whether or not an enzyme is involved in a particular catabolic pathway. It is important to note that the genes for DBF and DXN degradation are constitutively expressed or are constitutively expressed and slightly induced by DBF or
DXN. This is evidenced by whole cell enzyme assays performed over 25 years ago and by more recent raw data from RNA-seq experiments. The constitutive nature of DBF and DXN degradation in RW1 and the recruitment of a chromosomal gene for the second step of the DXN pathway highly suggests that the ability to degrade one or both of these substrates has recently evolved. Our experiments strongly point in this direction because when pSWIT02 dbfB was replaced with the chromosome SWIT3046 gene the new strain could grow on both DXN and DBF. A more highly evolved organism would already have SWIT3046 moved to the pSWIT02 plasmid so that the genes could conjugate as a unit into a different bacterium and bring an essential gene for DXN degradation with it.

One question that remains is What is the natural substrate for SWIT3046? Based on the genome sequence (accession version number CP000699.1), SWIT3046 is in a potential operon with orfs annotated (in order) as a TonB-dependent receptor, a phytanoyl-CoA dioxygenase, SWIT3046, an FAD containing monoxygenase, a second TonB-dependent receptor, and a sulfatase. The operon is preceded by a transposon and some transposons are known for activating adjacent genes by read through of a promoter. Upstream and oriented in the opposite direction is an operon potentially encoding a dioxygenase and a gentisate pathway and contains the dxnB2 gene encoding a well-studied hydrolase (101). It is possible that the natural substrate of SWIT3046 is the enzymatic product of the adjacently encoded monoxygenase. What this natural substrate is remains unknown.

Our work explains why DXN degrading bacteria are rarely found in nature compared to DBF degrading bacteria. The RW1 enzymes for DBF degradation are encoded by the potentially transmissible plasmid pSWIT02 that is found in other DBF
(but not DXN) degrading organisms (*Sphingomonas* sp. strain HH69 (63, 96) for
instance). The enzymes encoded by pSWIT02 are not sufficient for degradation of DXN.
A simple BLASTP search with SWIT3046 shows that this enzyme is only found in *S.
wittichii* strains (above 97% identity while the next best matches are below 60% identity).
Once pSWIT02 is in a host like RW1 with an appropriate SWIT3046-like ring cleavage
enzyme then a DXN degrading strain is formed. Thus, both plasmid and chromosomal
encoded enzymes are needed for RW1 to degrade DXN.
Figure 3. Growth of RW1, RW1ΔdbfB, RW1Δ3046, and RW1ΔdbfBΔ3046 on dibenzofuran.
Figure 4. Growth of RW1, RW1ΔdbfB, RW1Δ3046, and RW1ΔdbfBΔ3046 on dibenzo-\textit{p}-dioxin.
Figure 5. Dendrogram of RW1 meta ring cleavage enzymes with selected reference enzymes.

The ABQ number is the protein ID and the SWIT number is the gene ID in the GenBank database.
### Table 1. Table of transcriptomic and proteomic data.

The right three columns are expression ratios reported in the literature while the left three columns are extracted from the raw data of the RNA-Seq paper.

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<th>DD/SU</th>
<th>DF/SU</th>
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Figure 6. Growth of the complemented knockout mutants on dibenzofuran.
Figure 7. Growth of the complemented knockout mutants on dibenzo-\textit{p}-dioxin.
Figure 8. Schematic representation of the dbfB gene replacement constructs.
Figure 9. Schematic representation of the SWIT3046 gene replacement constructs.
Figure 10. Growth of gene replacement constructs on dibenzofuran.
Figure 11. Growth of gene replacement constructs on dibenzo-\(p\)-dioxin.
Figure 12. Metabolic map showing the role of DbfB and SWIT3046 in dibenzofuran and dibenzo-\(p\)-dioxin degradation.
Chapter 3: Growth of *Sphingomonas wittichii* RW1 on Dibenzo-*p*-dioxin

Requires a Chromosomally Encoded Hydrolase

**Introduction**

*Sphingomonas wittichii* RW1 was isolated from the Elbe River in northern Germany for its ability to grow on both dibenzofuran (DBF) and dibenzo-*p*-dioxin (DXN) as the sole source of carbon and energy (21). RW1 metabolizes DBF and DXN by similar catabolic pathways initiated by an angular dioxygenase complex system that dihydroxylates one of the aromatic rings forming a highly unstable intermediate that spontaneously decomposes to 2,2′,3-trihydroxybiphenyl (THB) and 2,2′,3-trihydroxybiphenyl ether (THBE) respectively (21, 50). A *meta* cleavage enzyme cleaves the hydroxylated ring (52) to form 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate (2HP-HOPDA) from THB and 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate (2HP-O-HOPDA) from THBE (53, 54). The alkene chain of the ring cleavage products is cleaved by a hydrolase forming salicylate (DBF pathway) and catechol (DXN pathway).

While aromatic hydroxylating dioxygenases are key enzymes in initiating the degradation of aromatic compounds, hydrolases are often a bottleneck in the degradation pathway (85, 102, 103). This is especially true for the degradation of compounds with more than one aromatic ring and for chlorinated compounds such as PCBs. Based on substrate specificity, bacterial hydrolases can be classified into three groups. Hydrolases belong to group I and II that are involved in bicyclic and monocyclic aromatic hydrocarbons and group III those are involved in hetero aromatic hydrocarbons biodegradation (104). Due to their strict substrate specificity (76, 86), hydrolases limit the
degradation of many aromatic hydrocarbons and their chlorinated substituents. An example is the BphD hydrolase from *Burkholderia* sp. strain LB400 that is a key enzyme in the biodegradation of many PCBs (87). Another important hydrolase is CarC from the carbazole degrading *Pseudomonas resinovorans* CA10 that cleaves metabolites from both carbazole and DBF (105). One important example of the importance of hydrolases is a comparison of the *P. putida* F1 toluene degradation pathway and the *Burkholderia* sp. strain LB400 biphenyl degradation pathway. While the three initial enzymes in the F1 toluene pathway are capable of metabolizing biphenyl to HOPDA, the bottleneck for growth on biphenyl is the TodF hydrolase. Addition of the LB400 BphD hydrolase to F1 overcomes this bottleneck (85, 106). The catalytic mechanism of aromatic pathway hydrolases has been extensively examined (54, 86, 87, 89, 101, 107-113). The enzyme specificity is due to the conserved catalytic triad (nucleophile-acid-histidine) found in all alpha/beta superfamily meta cleavage product hydrolases where the nucleophile is always a serine (107, 110, 113).

Two isofunctional hydrolases (H1/DxnB1/SWIT4895 and H2/DxnB2/SWIT3055) have been purified from *S. wittichii* RW1 (53) grown on salicylate. Both of these enzymes hydrolyze 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate [HOPDA] (biphenyl metabolite) and 2HP-HOPDA (DBF metabolite) to benzoate and salicylate, respectively. The two enzymes belong to the class III meta cleavage product hydrolases (54) but are monomeric (53) whereas other aromatic hydrolases are multimeric (86, 89, 114). No information has been published on their activity toward 2HP-O-HOPDA. The difference between 2-HP-HOPDA (from DBF) and 2-HP-O-HOPDA (from DXN) is the oxygen atom between the ring and the six carbon side chain. DxnB2 has been extensively
studied and is known to hydrolytically cleave both C-C and C-O bonds (54, 111).

Interestingly, \textit{dxnB1} (SWIT4895) is localized to pSWIT02 in RW1 and is in the main DXN degradation locus (\textit{dxnA1A2B1Cfdx3}) between the genes encoding the large and small subunits of the oxygenase component (\textit{dxnA1A2}) and the ferredoxin component (\textit{fdx3}) of the DBF/DXN dioxygenase. The \textit{dxnB2} (SWIT3055) gene, on the other hand, is localized to the chromosome.

It is assumed that the DxnB1 and DxnB2 hydrolases are involved in the ability of RW1 to grow on both DXN and DBF. However, this is based on the activities of the purified enzymes and the constitutive nature of the cognate genes. By our count the RW1 genome encodes 35 possible aromatic pathway hydrolases and an examination of raw RNA-seq data (91) shows that three of these (SWIT0910, SWIT3055, and SWIT4895) are constitutively expressed. It is our hypothesis that all three of these constitutively expressed hydrolases are involved in RW1 DXN and/or DBF degradation. In the present work we used a combination of gene knockout and physiological experiments to determine the role, if any, of each of these three hydrolases in RW1 DXN and DBF degradation.

**Materials and Methods:**

**Bacterial strains, plasmids, media and growth conditions.** \textit{Sphingomonas wittichii} RW1 is the wild type strain capable of utilizing dibenzofuran and dibenzo-\textit{p}-dioxin as the sole carbon and energy source (21). \textit{E. coli} DH5\(\alpha\) was the recipient strain in all cloning experiments. The pGEM-T easy vector (Promega, Madison, WI) was used to clone PCR products. pRK415 (97) was used to construct clones for gene knockout and for complementation experiments. Mineral salts basal medium [MSB] (98) was used for
carbon source and metabolite accumulation studies. When needed, L-phenylalanine was added to MSB at 10 mM final concentration. DBF and DXN were dissolved in acetone in a sterile flask to a calculated final concentration of 3 mM and the flasks left in a fume hood for 5 to 6 hours to allow for complete evaporation of the acetone. After addition of MSB medium the flasks were sonicated in a Branson 1800 water bath sonicator for 5 minutes to disperse the substrate crystals throughout the medium. DBF and DXN crystals were added on the lid of the MSB agar petri dish for growth tests as the sole carbon source. Amberlite IRA-400 Chloride resin (Sigma-Aldrich, St. Louis, MO) was added at 2 mg/ml MSB broth when needed. LB agar and LB broth were used as complete media. RW1 wild type and all mutants were grown aerobically at 30°C and *E. coli* strains were aerobically cultured in LB medium at 37°C. Ampicillin, gentamicin, kanamycin, and tetracycline were added to the medium when needed at 100, 15, 50, and 15 µg/ml, respectively.

**DNA Techniques.** Total genomic DNA from RW1 and RW1-derived strains was prepared using the Qiagen (Germantown, MD) Ultra Clean Microbial Kit and plasmid DNA was purified using the Macherey-Nagel (Bethlehem, PA) NucleoSpin Plasmid Kit following the manufacturers’ instructions. Transformation of plasmid DNA into competent *E. coli* strains was performed by the calcium chloride-glycerol transformation procedure (99). PCR products and restriction fragments were purified using the GeneClean III Kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions. Restriction enzymes and ligations of DNA samples were performed as recommended by the supplier (New England Biolabs, Ipswich, MA). All PCR amplifications were performed with the NEB Phusion High-Fidelity kit when the genes
were needed for downstream applications and with NEB Taq when used for mapping and screening purposes following instructions from the supplier. All PCR generated fragments used for downstream applications were sequenced to verify that no base changes were introduced. DNA sequencing was conducted by Genewiz (South Plainfield, NJ).

**Construction of hydrolase knockout mutants and complementation.** The three hydrolases were PCR amplified from RW1 gDNA using the Phusion High Fidelity PCR Kit (NEB, Ipswitch, MA). The *dxnB1* gene was PCR amplified with flanking regions of 0.60 kb upstream of the gene start codon and 0.47 kb downstream of the gene stop codon using the primers GGGGAATTCCgaagagctcgaacctgcag and GGGGAATTCCgaagagctcgaacctgcag containing *Eco*RI restriction site on both ends. The resulting 1.92 kb DNA fragment was ligated into pGEM7Z (Promega, Madison, WI) after digestion with *Eco*RI to form pGEM7_dxnB1. The p34S-Km3 (100) kanamycin cassette was digested with *Sal*I and ligated into similarly digested pGEM7_dxnB1 to form pGEM7_dxnB1-km. The latter plasmid was digested with *Eco*RI and ligated into similarly digested pRK415 to make the final construct pRK_dxnB1KO-km and transformed into the competent *E. coli* DH5α.

SWIT3055 (*dxnB2*) was PCR amplified with flanking regions of 0.54 kb upstream of the gene start codon and 0.42 kb downstream of the gene stop codon with the primers GGAAGCTTctgggtacgctgctgc and GGGTCTAGAcctggctgctgc containing *Hind*III and *Xba*I restriction sites, respectively. The resulting 1.8 kb fragment was TA cloned into pGEM-T Easy (Promega, Madison, WI) to form pGEMT_3055. A gentamicin cassette was used to disrupt *dxnB2* in the unique *Bcl*I site after digestion of
p34S-Gm (100) with BamHI (compatible end with BciI) to form pGEMT_3055-gm. The latter construct was digested with HindIII and XbaI and ligated into similarly digested pRK415 to form the final construct pRK_3055KO-Gm and transformed into DH5α.

The third hydrolase, SWIT0910, was PCR amplified with flanked regions of 0.70 kb upstream of the gene start codon and 0.42 kb downstream of the gene stop codon with the primers GGAAGCTTgcaacatcgtcctggtcg and GGAATTCgcagggcataagcgacgcagtc containing HindIII and EcoRI restriction sites, respectively. The resulting 1.89 kb fragment was purified and digested with HindIII and EcoRI and ligated into similarly digested pET30a (Sigma, St. Louis, MO) to form pET_0910. A gentamicin or kanamycin cassette was used to disrupt SWIT0910 in the unique SalI site after digestion of pET0910 and the antibiotic resistance cassette with SalI to form pET_0910-Gm or pET_0910-Km. The latter constructs were digested with HindIII and EcoRI and ligated into similarly digested pRK415 to form the final constructs pRK_0910KO-Gm or pRK_0910KO-Km. The final knock out constructs were transferred by triparental mating using the helper pRK2013 into RW1 with selection on MSB supplemented with phenylalanine and tetracycline and knockouts constructed as described in Chapter 2.

SWIT0886 is nearly identical to SWIT0910. A 0.83 kb fragment of SWIT0886 was PCR amplified using the primers GGTTCTAGAcccagggcagcctgcctgc and GAATTCgacgatggcggtttcatcgcg containing XbaI and EcoRI restriction sites, respectively. The PCR product was purified and cloned into the pGEM-T vector to form pGEMT_0886. The gene was removed from pGEM_0886 with XbaI and EcoRI and ligated into similarly digested pRK415 to form pRK_0886. The final construct was
transferred into RW1Δ0910 by triparental mating and transconjugants selected on MSB supplemented with phenylalanine and tetracycline.

Complementation of the mutations was performed by cloning the corresponding gene into pRK415 under lac promoter expression. Gene cassettes for the three hydrolase genes were constructed by cloning appropriate PCR fragments into the TOPO pCR2.1 vector (Sigma, St. Louis, MO). Forward primers incorporated an EcoRI or a SalI site and reverse primers incorporated a HindIII site. A 0.87 kb dxnB1 PCR fragment was amplified using the primers

GGGAATTCCGGGAATCGTGAGGATAGAAATGACCCAGC and

CCCAAGCTTGCATGCTAGAATTTCCGAGCG, a 0.82 kb PCR fragment containing SWIT0910 was amplified using the primers GAATTCCgaggacggatcggatcgc and

AAGCTTatcgctggcagggaggt, and a 0.89 kb PCR fragment containing SWIT3055 was amplified using the primers GTCGACgacggcattgccggtcggt and

AAGCTTcggcccatgcctcag. The three hydrolases were digested with XbaI and KpnI from pCR_dxnB1, pCR_3055, and pCR_0910, gel purified, and ligated into similarly digested pRK415 to form pRK_dxnB1, pRK_3055, and pRK_0910, respectively. The resulting constructs were transferred into the mutant strains by triparental mating with selection on MSB supplemented with phenylalanine and tetracycline. The presence of the constructs in RW1 mutants were verified by PCR.

**Growth curves.** RW1 and mutant strains were incubated overnight on LB agar with the appropriate antibiotic at 30°C. LB broth (50 ml) was inoculated with a loopful of cells and incubated over night at 30°C in a rotary shaker (180 rpm). MSB broth (50 ml) with phenylalanine was inoculated from the LB broth culture with a starting OD at
600nm of 0.05 and incubated at 30°C in a rotary shaker (180 rpm). The strains were
grown in MSB phenylalanine twice and when the optical density of the cells reached 2.0
in the second culture triplicate MSB broth cultures containing either 3 mM DBF or 3 mM
DXN were inoculated from each strain and OD readings taken at specific times. All
growth curves were plotted using the Prism software (GraphPad, San Diego, CA).

**Results**

**DxnB1 (SWIT4895) and DxnB2 (SWIT3055) function in DBF degradation but not
DXN degradation**

Bunz and Cook (53) previously isolated two isofunctional hydrolases active on
HOPDA (biphenyl metabolite) and 2HP-HOPDA (DBF metabolite). Comparison of the
N-terminal sequence of these two enzymes to the completed genome sequence (43)
identified the genes as SWIT4895 (for H1/DxnB1) and SWIT3055 (for H2/DxnB2). In
order to identify the role of each of these enzymes in DBF and DXN metabolism we
targeted these genes for knockout mutagenesis. As expected, RW1ΔdxnB1 and
RW1ΔdxnB2 grew at the same rate and extent as the wild type RW1 on DBF and DXN
as the sole carbon source (Figure 13 and Figure 14). This data suggests that the enzymes
are truly isofunctional under physiological conditions since both single knockouts grew
normally. The double knockout strain RW1ΔdxnB1ΔdxnB2 did not grow on DBF
(Figure 15) further demonstrating that the two hydrolases equally contribute to the
third enzymatic step in DBF degradation and that no other RW1 hydrolase functions in
this step of the pathway. Interestingly, the double knockout RW1ΔdxnB1ΔdxnB2 grows
on DXN (Figure 16) at the same rate and extent as the wild type RW1 showing that a
third hydrolase must function in the DXN pathway and that this third hydrolase does not function in the DBF pathway.

**Identification of a third hydrolase functional in DXN but not DBF degradation**

By our count the RW1 genome sequence contains genes encoding 35 potential aromatic compound pathway hydrolases (Figure 17). There have been multiple transcriptomic and proteomic studies examining RW1 during growth on DXN, DBF, and related compounds (59, 78, 90, 91). However, transcriptomic and proteomic studies typically report differences (ratios) in gene expression between growth on one substrate versus another. Since the RW1 DXN and DBF catabolic pathways are constitutively expressed (21) we examined the raw RNA-seq data from a transcriptomic study comparing DXN, DBF, and succinate grown RW1 (91). As expected, $dxnB1$/SWIT4895 and $dxnB2$/SWIT3055 are constitutively expressed with some slight variation (no more than 3x) between the three growth substrates (Table 2). In addition, the SWIT0886 and SWIT0910 genes, encoding potential aromatic pathway hydrolases, were constitutively expressed. Raw SWIT0886 and SWIT0910 RNA-seq counts were about the same for growth on succinate and DXN but were down by approximately half for growth on DBF. Interestingly, SWIT0886 and SWIT0910 are almost identical to each other with 13 internal base pair differences out of 753 bases (changing only three amino acids) plus a nine base "tail" on SWIT0910 (Figure 18). Given that the two genes are 98.27% identical and that many RNA-seq software programs incorrectly assign RNA-seq reads to multiple gene copies we re-analyzed the raw RNA-seq data in Chai et al. (91) for genes SWIT0886 and SWIT0910. RNA-seq reads matching SWIT0886 and/or SWIT0910 were binned into three categories: matching both SWIT0886 and SWIT0910, matching
only SWIT0886, and matching only SWIT0910. The results (Chai and Zylstra, personal communication) show that SWIT0886 was not expressed on any of the three growth substrates (SWIT0886 only were <0.15% of the total SWIT0886+SWIT0910 reads) and that SWIT0910 was expressed on all three growth substrates (SWIT0910 only were 23% to 33% of the total SWIT0886+SWIT0910 reads). We therefore targeted SWIT0910 for gene knockout to examine its role in RW1 DXN and DBF metabolism. RW1Δ0910 grows on DBF as the sole carbon source at the same rate and extent as the wild type strain RW1 (Figure 13). This fact, coupled with the fact that RW1ΔDxnB1ΔDxnB2 does not grow on DBF indicates that SWIT0910 plays no role in DBF degradation by RW1. Surprisingly, however, RW1Δ0910 does not grow on DXN (Figure 14). This data proves that SWIT0910 is the only RW1 hydrolase involved in growth of RW1 on DXN. The DBF pathway hydrolases DxnB1/SWIT4895 and DxnB2/SWIT3055 cannot take the place of SWIT0910 during growth on DXN even though they are constitutively expressed (Raw RNA-seq reads indicate 1.5x to 4.0x higher numbers for SWIT4895 and SWIT3055 respectively during growth on DXN vs DBF). We also constructed the double mutant strains RW1ΔDxnB1Δ0910 and RW1ΔDxnB2Δ0910 with the expected results: both double knockout strains do not grow on DXN but do grow normally on DBF (Figure 15 and Figure 16).

**Complementation**

In order to prove that the growth experiments described above are due to the specific gene knockout and not due to an effect on downstream genes complementation experiments were performed. For the double knockout RW1ΔdxnB1ΔdxnB2 addition of either plasmid pRK_dxnB1 or pRK_dxnB2 restored wild type growth on dibenzofuran
(Figure 19) indicating that lac promoter mediated expression of either of these two genes was sufficient to provide enough enzyme for complementation and that the knockout mutation only affected the dxnB1 or dxnB2 gene. For the knockout RW1Δ0910 complementation to wild type growth on dioxin (Figure 20) was achieved not only with the pRK_0910 plasmid but also the pRK_0886 plasmid. As mentioned above, SWIT0886 and SWIT0910 are almost identical but SWIT0886 is not expressed when RW1 is growing with succinate, DXN, or DBF as the carbon source. This means that both enzymes, despite their minor amino acid sequence differences, are capable of cleaving 2HP-O-HOPDA.

**Discussion:**

The work presented here shows the value and culmination of a multifaceted approach to aromatic hydrocarbon degradation by a number of researchers over the last 27 years. *S. wittichii* RW1 was isolated in 1992 for the ability to grow on DBF and DXN (21). Two aromatic pathway hydrolases were purified (53), characterized in depth (54, 101, 113), and their genes identified (43). The underlying physiology of RW1 DXN and DBF degradation has been probed using transcriptomic (90, 91), proteomic (59, 78), and Tn-seq (115) methodologies. In the present work we established that three different meta cleavage product hydrolases are involved in DXN and DBF degradation. Two of these hydrolases, DxnB1/SWIT4895 and DxnB2/SWIT3055 were previously isolated for the ability to cleave HOPDA (biphenyl pathway) and 2HP-HOPDA (DBF pathway). Surprisingly, neither of these two enzymes is capable of functioning in the RW1 DXN pathway and both enzymes contribute equally to the RW1 DBF pathway. The single knockouts RW1ΔDxnB1 and RW1ΔDxnB2 grow normally on DBF and the double
knockout RW1ΔDxnB1ΔDxnB2 does not grow on DBF. All three knockout mutant strains grow on DXN. Using a combined genomic and transcriptomic approach SWIT0910 was postulated to be the DXN pathway hydrolase and a strain (RW1Δ0910) knocked out for this gene did not grow on DXN and grew normally on DBF. Based on these facts we postulate that SWIT0910 has little or no activity toward the DBF metabolite 2HP-HOPDA and that DxnB1/SWIT4895 and DxnB2/SWIT3055 have little or no activity toward the DXN metabolite 2HP-O-HOPDA. Since the only difference between these two compounds is the oxygen linking the aromatic ring to the side chain, the oxygen must play a significant role in the ability (or inability) of the three hydrolases to cleave the compound. Based on this hypothesis it is not surprising that the SWIT0910 hydrolase enzyme wasn't isolated by Bunz and Cook (53) since they did not screen using 2HP-O-HOPDA as the substrate. The three enzymes are sufficiently different from each other with DxnB1 and DxnB2 showing 44% amino acid identity over the full length of the protein. However SWIT0910 shows less than 30% amino acid identity to DxnB1 and DxnB2 but only in the N-terminal half of the protein; there is no detectable identity in the C-terminal half of the protein. The DxnB2 enzyme has been extensively studied (54, 101, 111-113) and the hydrolase catalytic triad (nucleophile-acid-histidine) identified as Ser105, Asp227, His255. Interestingly, an alignment (Figure 21) of the three enzymes DxnB1, DxnB2, SWIT0886, and SWIT0910 shows that Ser195 and Asp227 of DxnB2 are conserved in the other three enzymes but that the His225 is only conserved in DxnB1 but not in SWIT0886 and SWIT0910. Since the His225 is part of the catalytic triad in DxnB2 another amino acid must take its place in SWIT0886 and SWIT0910.
Sphingomonads are well known for their ability to degrade a large number of compounds. This is correlated by the fact that their genomes encode many different degradative enzymes (43, 116) whose genes are not often organized in the typical operonic structure (55, 117). Soil organisms such as *S. wittichii* RW1 are constantly evolving to take advantage of changing environmental conditions and growth substrates. To our knowledge there are only three bacterial strains known to grow on DXN (21, 92, 93). *S. wittichii* is the only one of these three organisms that has been extensively studied. In contrast to DXN degradation, many strains have been isolated for the ability to degrade DBF and many of these strains can partially metabolize DXN after growth (induction) on DBF. In RW1 the genes encoding the upper pathway for DBF degradation are located in multiple locations on the plasmid pSWIT02. This plasmid is also found in other strains that can degrade DBF but not DXN (95, 96). We previously showed (Chapter 2) that DXN degradation absolutely depends on a THBE meta ring cleavage enzyme encoded by the chromosome. The DBF degradation pathway THB meta ring cleavage enzyme encoded by pSWIT02 does not function for THBE ring cleavage to allow RW1 growth on DXN. Now, in the current chapter, an analogous situation is discovered. There are two RW1 DBF degradation pathway 2HP-HOPDA hydrolases, one encoded by the chromosome and one encoded by pSWIT02 (Figure 22). They are equally active in the catabolic pathway, deletion of one or the other does not affect RW1 growth on DBF. However, the DXN degradation pathway 2HP-O-HOPDA hydrolase is encoded by the RW1 chromosome and is absolutely required for growth (Figure 22). Thus, the mystery of why RW1 is the only DXN degrading organism is solved. The DXN catabolic pathway requires an initial ring hydroxylating dioxygenase encoded by
pSWIT02. The remaining enzymes in the upper catabolic pathway, the THBE meta
cleavage dioxygenase and the 2HP-O-HOPDA hydrolase are encoded by the
chromosome. In order to evolve an organism that grows on DXN nature needs to
combine the pSWIT02 plasmid with the appropriate host organism.
Figure 13. Single hydrolase gene knockout growth curves on dibenzofuran.
Figure 14. Single hydrolase gene knockout growth curves on dibenzo-\(p\)-dioxin.
Figure 15. Double hydrolase gene knockout growth curves on dibenzofuran.
Figure 16. Double hydrolase gene knockout growth curves on dibenzo-\(p\)-dioxin.
Figure 17. Dendrogram of RW1 hydrolase enzymes.

The ABQ number is the protein ID and the SWIT number is the gene ID in the GenBank database. Enzymes known as DxnB/DxnB1 and DxnB2 are indicated.
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Table 2. RNA-Seq raw data for RW1 hydrolase genes.

Constitutive genes are highlighted in yellow.
Figure 18. Comparison of SWIT0886 (top) with SWIT0910 (bottom).

Identical nucleotides are indicated by a dash in the SWIT0910 sequence. Different nucleotides and the corresponding different amino acids are indicated in red.
Figure 19. Complementation of hydrolase knockout mutant growth curves on dibenzofuran.
Figure 20. Complementation of hydrolase knockout mutant growth curves on dibenzo-\textit{p}-dioxin.
Figure 21. Alignment of the DxnB1/SWIT4895, DxnB2/SWIT3055, SWIT0886, and SWIT0910 hydrolases.

Conserved amino acid residues are shaded in green. The catalytic triad S105 H255 D227 of DxnB2 are indicated by red arrows.
Figure 22. Metabolic map showing the role of DxnB, SWIT3055, and SWIT0910 in dibenzofuran and dibenzo-\(p\)-dioxin degradation.
Chapter 4: Genetic Manipulation of the Second Enzymatic Step in the

*Sphingomonas wittichii* RW1 Dibenzo-p-dioxin and Dibenzofuran Pathways

**Introduction**

Microorganisms play a major role in the carbon cycle through mineralization of the many aromatic xenobiotic and natural compounds in the environment. Many microorganisms have been isolated for their capability to aerobically degrade polycyclic aromatic environmental pollutants such as dibenzofuran (DBF) (20, 25, 27, 40, 41, 118), biphenyl (119, 120) and even dibenzo-p-dioxin (21). In general, there are three stages in the upper pathway for simple polycyclic compounds. In stage 1 an angular or lateral dioxygenase attacks one aromatic ring forming a *cis*-dihydrodiol compound. For an angular dioxygenase attack the resulting product is an unstable hemiacetal which spontaneously converts to the corresponding doubly hydroxylated compound. For a lateral dioxygenase attack the resulting *cis*-dihydrodiol is converted by a dehydrogenase enzyme to a doubly dihydroxylated compound. In stage 2 the ring hydroxylated compound undergoes meta ring cleavage and in stage 3 a hydrolase separates what is left of the first aromatic ring from the remaining aromatic ring(s).

Extradiol (meta) ring cleavage dioxygenases are a family of ferrous iron containing multimeric enzymes (32). The enzymes cleave between one hydroxylated carbon atom and an adjacent non-hydroxylated one and do not require any external reductant. Variants of ring cleavage enzymes with different substrate ranges and enzymatic activities are found in a wide range of aromatic catabolic pathways including those for toluene, benzene, biphenyl, naphthalene, dibenzofuran, and dibenzo-p-dioxin (32, 52, 75, 121, 122). Genetic studies on the TOL pathway showed that the TOL
extradiol dioxygenase is a major determinant of substrate specificity for this pathway (74, 123). Several studies have shown the critical role of extradiol dioxygenases in PCB degradation due to the various type of inhibition of this type of enzyme and incapability to transform certain chlorinated 2,3-dihydroxybiphenyls [DHB] (76, 102) which often result in suicide inactivation (124). For example, ortho chlorinated biphenyl metabolites strongly inhibit and promote a suicide inactivation of the 2,3-dihydroxybiphenyl 1,2-dioxygenase in *Burkholderia* sp. strain LB400 (68).

We recently showed (Chapter 2) that the dibenzofuran (DBF) and dibenzo-\(p\)-dioxin (DXN) degrading strain *Sphingomonas wittichii* RW1 contains two constitutively expressed extradiol dioxygenases, DbfB/SWIT4902 and SWIT3046. Both enzymes function in the DBF pathway for cleavage of 2,2',3-trihydroxybiphenyl (THB) but only the latter enzyme functions in the DXN pathway for cleavage of 2,2',3-trihydroxybiphenyl ether (THBE). This shows the importance of substrate specificity in the ability of an enzyme to physiologically function in a catabolic pathway. We were intrigued by the fact that these two related enzymes had such dramatically different activities: the one enzyme could efficiently cleave both substrates while the other enzyme could only efficiently cleave the one substrate. We wondered how common THB and especially THBE ring cleavage activity is among DHB extradiol dioxygenases. The difference between DHB and THB is a hydroxyl group on the non-cleaved aromatic ring and the difference between THB and THBE is an oxygen atom connecting the two rings together. Several DHB ring cleavage enzymes are known with those from *Burkholderia* sp. strain LB400 (biphenyl degradation), *Sphingobium yanoikuyae* B1 (polycyclic degradation), and *Pseudomonas putida* F1 (toluene degradation) being among the best
studied enzymes of this class (76, 103, 106, 125, 126). The three enzymes are related to many of the corresponding meta ring cleavage enzymes in RW1, especially with DbfB and SWIT3046 (Figure 23). In the present work we analyzed whether LB400 BphC, B1 BphC, and F1 TodE could replace RW1 DbfB1 and/or SWIT3055 for growth on DBF and DXN.

**Materials and Methods:**

**Bacterial strains, plasmids, media and growth conditions.** *Sphingomonas wittichii* RW1 is the wild type strain capable of utilizing dibenzofuran and dibenzo-p-dioxin as the sole carbon and energy source (21). *Burkholderia* sp. strain LB400 (120) and *Sphingobium yanoikuyae* B1 (119) are the wild type strains capable of utilizing biphenyl as a sole source of carbon and energy. *Pseudomonas putida* F1 is the wild type strain capable of utilizing benzene and toluene as the sole source of carbon and energy (127). *E. coli* DH5α was the recipient strain in all cloning experiments. The pGEM-T easy vector (Promega, Madison, WI) was used to clone PCR products. pRK415 (97) was used to construct clones for gene knockout and for complementation experiments. Mineral salts basal medium [MSB] (98) was used for carbon source and metabolite accumulation studies. When needed, L-phenylalanine was added to MSB at 10 mM final concentration. DBF and DXN were dissolved in acetone in a sterile flask to a calculated final concentration of 3 mM and the flasks left in a fume hood for 5 to 6 hours to allow for complete evaporation of the acetone. After addition of MSB medium the flasks were sonicated in a Branson 1800 water bath sonicator for 5 minutes to disperse the substrate crystals throughout the medium. DBF and DXN crystals were added on the lid of the MSB agar petri dish for growth tests as the sole carbon source. Amberlite XAD7HPresin
(Sigma-Aldrich, St. Louis, MO) was added at 2 mg/ml MSB broth when needed. LB agar and LB broth were used as complete media. RW1, LB400, B1, and F1 wild type and all mutants were grown aerobically at 30°C and *E. coli* strains were aerobically cultured in LB medium at 37°C. Ampicillin, gentamicin, kanamycin, and tetracycline were added to the medium when needed at 100, 15, 50, and 15 µg/ml, respectively.

**DNA Techniques.** Total genomic DNA from *Sphingomonas wittichii* RW1, *Burkholderia* sp. strain LB400, *Sphingobium yanoikuyae* B1, and *Pseudomonas putida* F1 was prepared using the Qiagen (Germantown, MD) Ultra Clean Microbial Kit and plasmid DNA was purified using the Macherey-Nagel (Bethlehem, PA) NucleoSpin Plasmid Kit following the manufacturers’ instructions. Transformation of plasmid DNA into competent *E. coli* strains was performed by the calcium chloride-glycerol transformation procedure (99). PCR products and restriction fragments were purified using the GeneClean III Kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions. Restriction enzymes and ligations of DNA samples were performed as recommended by the supplier (New England Biolabs, Ipswich, MA). All PCR amplifications were performed with the NEB Phusion High-Fidelity kit when the genes were needed for downstream applications and with NEB Taq when used for mapping and screening purposes following instructions from the supplier. All PCR generated fragments used for downstream applications were sequenced to verify that no base changes were introduced. DNA sequencing was conducted by Genewiz (South Plainfield, NJ).

**Gene replacement construction.** The *dbfB* promoter is constitutively expressed (52, 55). In our previous work (Chapter 2), we showed that RW1 mutants that lack both
ring cleavage dioxygenases dbfB and SWIT3046 and have only SWIT3046 on the
plasmid pSWIT02 in the place of dbfB are capable of utilizing both DBF and DXN just
like the WT does. The constructs with F1 TodE, B1 BphC, and LB400 BphC were
constructed in a similar fashion.

LB400 bphC was inserted on RW1 pSWIT02 as following. A 1.05 kb region
upstream of the dbfB start codon including the dbfB ribosome binding site was amplified
by PCR using the primers GGAAGCTTcgttagcctctcagagtg (containing a HindIII site)
and ctgatgctcatgacgataactctcctttgc (containing overlap sequence with LB400 bphC). A
0.9 kb LB400 bphC fragment was PCR amplified using LB400 genomic DNA from the
bphC start codon without its ribosome binding site using the primers
gagttatcgtcatgagcatcagaagtttgg (containing overlap sequence with the end of the upstream
fragment) and cTCTAGAGctagctgttgctgcgc (containing a XbaI site). A 1.05 kb fragment
downstream of the dbfB gene was PCR amplified using the primers
CTCTAGAggccatacacctaacattgtc (containing a XbaI restriction site) and
CCGGATCCccacctcaagcgcatcctg (containing a BamHI restriction site). First, the
downstream fragment that contains the XbaI and BamHI restriction sites was cloned into
the pGEM-T vector, sequenced, and ligated into similarly digested pRK415 to make
pRK_Down. Second, the products of the upstream region and LB400 bphC were merged
by PCR overlap extension as following: the PCR products from two fragments were PCR
purified and mixed in one PCR reaction 1:1 ratio using Phusion HF PCR kit without
adding any primer under the following conditions: denaturation at 98°C for 10 sec,
anneal at 72°C for 40 sec, and extension at 72°C for 1 minutes for 10 cycles then the
following primers were added GGAAGCTTcgttagcctctcagagtg (the forward primer from
the upstream fragment with *HindIII* and cTCTAGAGtcagccgaggggaatatcc (the reverse primer for LB400 *bphC* containing a *XbaI* restriction site) and the reaction was returned to the PCR machine for another 25 cycles under the same conditions. The resulting 1.9 kb fragment was purified from an agarose gel and cloned into pGEM-T to yield pGEMT_UPLB400, transformed into DH5α, and sequenced. pGEMT_UPLB400 was digested with *HindIII* and *XbaI* and ligated into similarly digested pRK_Down to make pRK_LBrep. A gentamycin antibiotic cassette was digested from the p34S-Gm vector with *XbaI* and ligated into similarly digested pRK415_LBrep to make the final construct pRK_LBrep-Gm.

*B1 bphC* was inserted on RW1 pSWIT02 as following. A 1.05 kb upstream of *dbfB* start codon including the *dbfB* ribosome binding site was amplified by PCR using the primers GGAAGCTTcgttagcctctcacgagtg (containing *HindIII* restriction site) and ctgctaccatgacgataactctccttttgc (containing overlap sequence with *B1 bphC*). A 0.9 kb *B1 bphC* fragment was PCR amplified using B1 genomic DNA from the gene start codon without its ribosome binding site using the primers gagttatcgtcatggtagcagtcacggaac (containing overlap sequence with the end of the upstream fragment) and cTCTAGAGtcagccgaggggaatatcc (containing a *XbaI* site). The upstream and B1 *bphC* fragment were ligated by PCR overlap extension as following: the PCR products from two fragments were PCR purified and mixed in one PCR reaction 1:1 ratio using Phusion HF PCR kit without adding any primer under the following conditions: denaturation at 98°C for 10 sec, anneal at 72°C for 40 sec, and extension at 72°C for 1 minutes for 10 cycles then the following primers were added GGAAGCTTcgttagcctctctacgagtg (the forward primer from the upstream fragment-*HindIII*) and
cTCTAGAGtcagccgaggggaatatcc (the reverse primer for B1 *bphC* containing a *XbaI* site) and the reaction was returned to the PCR machine for another 25 cycles under the same conditions. The resulting 1.9 kb fragment was purified from an agarose gel and was cloned into pGEM-T to yield pGEMT_UPB1, transformed into DH5α, and sequenced. pGEMT_UPB1 was digested with *HindIII* and *XbaI* and ligated into similarly digested pRK_Down to make pRK_B1rep. A gentamycin antibiotic cassette was digested from p34S-Gm with *XbaI* and ligated into similarly digested pRK_B1rep to make the final construct pRK_B1rep-Gm.

F1 *todE* was inserted on RW1 pSWIT02 as following. A 1.05 kb upstream region of *dbfB* including the *dbfB* ribosome binding site was amplified by PCR using the primers GGAAGCTTcgttagcctctcacgagtg (containing a *HindIII* site) and gaatgctcatgacgataactctccttttgc (containing an overlap sequence with F1 *todE*). A 0.87 kb F1 *todE* fragment was PCR amplified using F1 genomic DNA from the gene start codon without its ribosome binding site using the primers gagttatcgtcatgagcattcaaagattgg (containing an overlap sequence with the end of the upstream fragment) and cTCTAGAGtcaggcgggcgcctg (containing an *XbaI* site). The upstream and F1 *todE* fragment were ligated by PCR overlap extension as following: the PCR products from two fragments were PCR purified and mixed in one PCR reaction 1:1 ratio using Phusion HF PCR kit without adding any primer under the following conditions: denaturation at 98°C for 10 sec, anneal at 72°C for 40 sec, and extension at 72°C for 1 minutes for 10 cycles then the following primers were added GGAAGCTTcgttagctctcagctag (the forward primer from upstream fragment with a *HindIII* site ) and cTCTAGAGtcagccgggccgctg (the reverse primer for F1 *todE* containing an *XbaI* site) and
the reaction was returned to the PCR machine for another 25 cycles under same conditions. The resulting 1.9 kb fragment was purified from an agarose gel and cloned into pGEM-T to yield pGEMT_UPF1, transformed into DH5α, and sequenced. pGE_UPF1 was digested with HindIII and XbaI and ligated into similarly digested pRK_Down to make pRK_F1rep. A gentamycin cassette was digested from p34S-Gm vector with XbaI and ligated into similarly digested pRK_F1rep to make the final construct pRK_F1rep-Gm.

The three final constructs pRK_LBrep-Gm, pRK_B1rep-Gm, and pRK_F1rep-Gm were transferred into RW1Δ3046 by triparental mating using the helper pRK2003. Knock out mutations were generated and screened as described earlier.

**Growth curves.** RW1 and its mutants were incubated overnight on LB agar with the appropriate antibiotic at 30°C. LB broth (50 ml) was inoculated with a loopful of cells and incubated over night at 30°C in a rotary shaker (180 rpm). MSB broth (50 ml) with phenylalanine was inoculated from the LB broth culture with a starting OD at 600nm of 0.05 and incubated at 30°C in a rotary shaker (180 rpm). The strains were grown in MSB phenylalanine twice and when the optical density of the cells reached 2.0 in the second culture triplicate MSB broth cultures containing either 3 mM DBF or 3 mM DXN were inoculated from each strain and OD readings taken at specific times. All growth curves were plotted using the Prism software (GraphPad, San Diego, CA).

**Results**

In our previous work (Chapter 2) we constructed the double knockout strain RW1ΔdbfBΔ3046 which is missing the two extradiol dioxygenases needed for DXN and DBF degradation. We also constructed RW1Δ3046ΔdbfB/3046 which is a double
knockout with SWIT3046 replacing \( dhfB \) which grows at the same rate and extent as the wild type RW1 on both DXN and DBF. Using the same general approach (summarized in Figure 24), the genes for three different meta cleavage dioxygenases were introduced into RW1ΔSWIT3046 so that the new strains lack both \( dhfB \) and SWIT3046 and either B1 \( bphC \), F1 \( todE \), or LB400 \( bphC \) are substituted for \( dhfB \) and thus under control of the constitutive \( dhfB \) promoter. The resulting strains RW1Δ3046ΔdbfB/B1bphC, RW1Δ3046ΔdbfB/F1todE, and RW1Δ3046ΔdbfB/LB400bphC were tested for their ability to grow on either DBF or DXN as the sole source of carbon and energy.

The data (Figure 25 and Figure 26) show that the LB400 BphC DHD ring cleavage enzyme efficiently attacks THB and THBE at a rate sufficient to allow RW1Δ3046ΔdbfB/LB400bphC to grow on DBF and DXN at the same rate and extent as the wild type strain RW1. The constructs RW1Δ3046ΔdbfB/B1bphC and RW1Δ3046ΔdbfB/F1todE did not grow on DXN and accumulated a brown colored compound in the culture media identified as THBE. Both RW1Δ3046ΔdbfB/B1bphC and RW1Δ3046ΔdbfB/F1todE grew on DBF but at rates much slower than RW1. Of the two constructs, RW1Δ3046ΔdbfB/F1todE grew the best, reaching stationary phase on DBF after 45 hours compared to the wild type RW1 which takes 15 hours under identical conditions to reach stationary phase. In contrast, RW1Δ3046ΔdbfB/B1bphC took over 120 hours to reach stationary phase (the growth curve was terminated before stationary phase was reached).

**Discussion**

In our limited survey of three different aromatic pathway extradiol dioxygenases we found that activities against THBE in the DXN pathway are less common than
activities against THB in the DBF pathway. This is most likely due to the similarity of
the THB structure to DHB (biphenyl pathway) and to 3-methylcatechol (toluene
pathway). THBE with the extra oxygen atom linking the aromatic part of the compound
to the second ring is somewhat recalcitrant to attack by the ring cleavage enzymes tested
in the current study. The genes for the toluene and biphenyl catabolic pathways in P.
putida F1 and Burkholderia sp. strain LB400 are related (85, 103, 106, 128) and therefore
it is not surprising that the F1 TodE and LB400 BphC enzymes were most similar in our
survey. For THB ring cleavage the LB400 BphC was just as efficient as the wild type
RW1 enzyme DbfB1 while the F1 TodE and B1 BphC enzymes were much less efficient
with very long doubling times of the engineered strains. It is not surprising that the B1
BphC enzyme had the least catalytic activity toward THB. The S. yanoikuyae B1 strain
is more adept at degrading polycyclic compounds like naphthalene or phenanthrene (129,
130) than Burkholderia sp, strain LB400. B1 is thus more of a polycyclic aromatic
hydrocarbon degrading strain and LB400 is more of a biphenyl and PCB degrading
strain. The "true" biphenyl degrading strain LB400 was also the only strain tested that
has a ring cleavage enzyme capable of cleaving THBE and its BphC enzyme fully
restored growth of the RW1ΔdbfBΔ3046 strain on DXN while no growth was seen with
the added F1 TodE and B1 BphC enzymes. Overall, our data bolsters the hypothesis that
the genes for DBF and DXN degradation evolved from the more common (more easily
found in nature) genes for biphenyl degradation.
Figure 23. Dendrogram showing the relationship of the B1 BphC, LB400 BphC, and F1 TodE meta cleavage enzymes to those in RW1.
Figure 24. Schematic representation of the meta ring cleavage gene replacements.
Figure 25. Growth curves on dibenzofuran of different ring cleavage enzymes cloned into an RW1 ring cleavage minus host.
Figure 26. Growth curves on dibenzo-\(p\)-dioxin of different ring cleavage enzymes cloned into an RW1 ring cleavage minus host.
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