NEW INSIGHT INTO THE CATABOLIC PATHWAY REDUNDANCIES

OF THE DIOXIN-MINERALIZING BACTERIUM

SPHINGOMONAS WITTICHII RW1

By

Suha Eleya

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Microbial biology

Written under the direction of

Dr. Gerben Zylstra

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2020

ABSTRACT OF THE DISSERTATION

New Insight into the Catabolic Pathway Redundancies of the Dioxin-Mineralizing

Bacterium *Sphingomonas wittichii* RW1

Suha Eleya

Dissertation Director:

Dr. Gerben Zylstra

Dioxins and dioxin-like compounds are highly toxic, ubiquitous polycyclic aromatic
hydrocarbons with important environmental and human health impacts. Dioxins are listed
among the most relevant environmental organic pollutants due to their high persistence
and extreme lipophilicity. In recent years, a number of bacterial strains have been isolated
and identified for their diverse metabolic capabilities to grow on and metabolize a broad
range of environmentally recalcitrant compounds including, dibenzo-*p*-dioxin and
dibenzofuran. Among these strains *Sphingomonas wittichii* RW1 has been one of the
most effective dioxin degraders studied so far. RW1 is of great interest for its diverse
metabolic activities and unusual genome structure. The complete genome sequence of
RW1 reveals that this strain contains one chromosome and two circular megaplasmids
referred to as pSWIT01 and pSWIT02. Many of the important catabolic genes that are
involved in dibenzofuran and dibenzo-*p*-dioxin degradation are located on the small
megaplasmid pSWIT02; particularly genes encoding the initial dioxygenase system
DxnA1A2, a ferredoxin Fdx3, and a reductase RedA2. This unusual dioxygenase system
initiates the oxygenolytic attack of dibenzofuran and dibenzo-*p*-dioxin in an angular

fashion. Here we report the first physiological identification of the important proteins that together function as the initial dibenzofuran and dibenzo-*p*-dioxin dioxygenase enzyme in RW1. Knock out mutagenesis showed that two reductases RedA1 and RedA2 and two ferredoxins Fdx1 and Fdx3 are interchangeable where either RedA1/RedA2 in combination with Fdx1/Fdx3 can function as an electron donor to the terminal oxygenase. The knockout mutants were also screened on substrates other than dibenzofuran and dibenzo-*p*-dioxin such as salicylate and benzoate. We discovered that the reductase RedA2 is involved in supplying electrons to the salicylate oxygenase since deletion of the *redA2* gene blocked the growth of RW1 on salicylate. Interestingly, single knockout mutants of *redA1* and *redA2* had no effect on RW1's ability to grow on benzoate while a double knockout mutant resulted in the loss of the ability to grow on benzoate. In addition, we constructed stable and unstable cloning vectors for sphingomonads based on the *Sphingobium yanoikuyae* B1 pKG2 plasmid and the *Sphingomonas wittichii* RW1 pSWIT02 plasmid. Stable vectors included the *rep* and *par* plasmid regions for replication and partitioning while unstable vectors included only the *rep* region. The stable vector pSEZ_RW1RP was used to cure RW1 of pSWIT02 by plasmid incompatibility. The cured strain RW1c was no longer able to grow on dibenzo-*p*-dioxin or dibenzofuran. A complementation test with the broad host range plasmid pRK415 carrying the initial dioxygenase system allows RW1c to grow on both dibenzo-*p*-dioxin and dibenzofuran. These results demonstrate that the only major role that pSWIT02 plays in the degradation of dibenzo-*p*-dioxin and dibenzofuran is to supply the initial dioxygenase responsible for the first step in the catabolic pathway.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: LITERATURE REVIEW

1.1. THE GENUS SPHINGOMONAS

Sphingomonads are a bacterial group of great interest to the bioremediation field due to their unusual capabilities to biodegrade and metabolize a wide range of monocyclic and polycyclic aromatic compounds including benzene, toluene, biphenyl, naphthalene, phenanthrene, pyrene, carbazole, xylenes, phenols, dibenzofurans, and dibenzo-*p*-dioxins (1-4). Some sphingomonad strains have even been found to grow on and utilize aromatic polymers such as lignin, a major component of plant cell walls (5-7). Sphingomonad species are well known for their significance in a wide variety of industrial applications and for their ability to produce exopolysaccharides called sphingans in particular the gellan polysaccharide, wellan polysaccharide, and rhamsan polysaccharide and also to produce carotenoids such as beta-carotene and nostoxanthis (8-12).

Sphingomonads were first classified by Takeuchi et al. in 2001 to comprise the four genera *Sphingomonas*, *Sphingobium*, *Novosphingobiu*m, and *Sphingopyxis* on the basis of 16S rRNA sequence analysis and fatty acid profiles (13-17). In 2002, Yabuuchi et al. re-classified the group, including *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, to involve only one genus called *Sphingomonas* (18). Sphingomonads are gram negative, yellow pigmented, strictly aerobic, chemoheterotrophs, rod-shaped bacteria that contain unique glycosphingolipids as a major cell envelope component in place of lipopolysccaride (13, 19).

Sphingomonads species are widely spread in nature, found both in terrestrial and sediment habitants and play key role in carbon cycling (13). They have been isolated from different habitats including, soil, air, the plant phyllosphere and rhizosphere, sediments, freshwater and aquatic environments (20-23). Moreover, some sphingomonad strains such as *Sphingomonas paucimobilis* have been isolated from hospital samples and equipment and found to be associated with human infections such as pneumonia, peritonitis, septicemia, meningitis, nosocomial infection and urinary tract infections (24, 25), and also have been implicated in biofilm formation (26). Several sphingomonad species have been proposed as potent metabolizers of a number of herbicides and pesticides in the environment (27).

The genus *Sphingomonas* was initially proposed by Yabuuchi et al. in 1990 (22, 28) on the basis of phylogenetic analysis and the presence of sphingoglycolipids (SGL) in the outer membrane instead of lipopolysaccharide found in other gram-negative bacteria (9, 28-31). Members of the genus *Sphingomonas* belong to the family Sphingomonadaceae in the alpha-4 subgroup of the alphaproteobacteria and are gram negative, obligate aerobes, motile rods, nonsporulating, chemoheterotrophic, nonfermentative, white or yellow pigmented bacteria (9, 17, 21, 22, 30, 32-34). Species of the genus *Sphingomonas* generally possess ubiqunone-10 (Q10) type respiratory quinone as a major respiratory quinone component (16, 35, 36). In addition to the biochemical and structural properties, sphingomonads are characterized distinct from other alphaprotobacteria by the unusual genetic organization of catabolic genes involved in aromatic compounds degradation that are often found scattered on the genome rather than clustered in a single transcriptional segment (37).

A number of *Sphingomonas* strains have been described for their versatile metabolic capabilities due to the presence of redundant aromatic pathways and multiple dioxygenase systems of broad substrate range specificity which enable these strains to utilize a numerous variety of polycyclic aromatic hydrocarbons and to play a crucial role in the elimination of herbicides and pesticides in the environment (38-41). Among this bacterial group, *Sphingomonas wittichii* RW1 has been considered to be the most efficient isolated dibenzofuran and dibenzo-*p*-dioxin degrader (31, 42). *S. wittichii* RW1 is of considerable interest for both the bioremediation and biotechnology fields due to its high potential to break down the backbone structure of dioxin and related compounds and to produce a number of important industrial products such as exopolysaccharides (43, 44). *S. wittichii* RW1 is also able to co-metabolize and aerobically transform mono and dichloro derivatives and other chlorinated congeners of dioxin and dioxin like compounds (45, 46).

The bacterium *S. wittichii* RW1 was initially isolated and described for the first time in 1992 by Wittich as a potent dioxin metabolizing bacterium (47, 48). The bacterium *S. wittichii* RW1 is ubiquitously found in soil in contaminated industrial sites with polycyclic aromatic hydrocarbons. The chemical composition of the outer membrane in *Sphingomonas wittichii* RW1 was found to be distinct from that of the outer membrane of other gram-negative bacteria as it possesses glycosphingolipids instead of lipopolysaccharides, a major component in the cell membrane of gram-negative bacteria (30, 49, 50). This unique property of *S. wittichii* RW1 helps to provide a hydrophobic environment in the outer membrane and therefore facilitates the uptake and degradation of dioxin and related compounds from the environment. The complete annotated genome

sequence of *S. wittichii* RW1 reveals that this strain, in addition to a main chromosome

(5,382,261 bp), contains two circular megaplasmids referred to as pSWIT01 (310,228 bp)

and pSWIT02 (222,757 bp)(1, 51), with DNA G+C content between 62% and 68% (30,

31). Additionally, the genome of *S. wittichii* RW1 possess a large number of transposase

genes flanking some of the important catabolic genes suggesting that these genes were

obtained through horizontal gene transfer (51). *S. wittichii* RW1 is characterized by the

unusual arrangement of the dioxin catabolic gene clusters where most of these genes are

located on variant loci on the megaplasmid and on the chromosome. For instance, the

multicomponent dioxin dioxygenase system is carried in one distinct locus of the small

megaplasmid. However, genes for the reductase RedA2 and the meta cleavage enzyme

Dbfb have been found to be located on a neighboring genomic segment on the

megaplasmid and oriented in the opposite direction to the dioxygenase system. Several

key enzymes that are implicated in the degradative pathways of the diaryl ethers dibenzo-

p-dioxin and dibenzofuran have been characterized and described in detail such as the

initial oxygenase, the extradiol dioxygenase, and the *meta*-cleavage product (MCP)

hydrolase (46). *S. wittichii* RW1 is well characterized both genetically and biochemically

and is considered to be a model organism that may facilitate the study of the molecular

mechanism of dioxin and other environmental toxicants metabolism (52). In addition, the

versatile metabolic capabilities of *S. wittichii* strain RW1 may provide promising

directions to the fields of bioremediation and biodegradation.

1.2. POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) comprise a large group of several

hundred types of recalcitrant, nonpolar, lipophilic, and toxic organic pollutants with

different molecular composition and structure (53-57). Polycyclic aromatic hydrocarbons (PAHs), including polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyls are known as persistent environmental pollutants and are among the most toxicants of concern at Superfund sites in the United States with over 600 PAH contaminated sites listed on the United States Environmental Protection Agency National Priorities List (58-63). These compounds are formed and released into the environment by anthropogenic sources such as industrial contamination, the chemical synthesis of pesticides and herbicides, gasification and incomplete combustion of organic compounds or natural sources such as volcano eruptions and forest fires (37, 64-68).

The chemical backbone of PAHs is composed of two or more fused aromatic rings (69, 70). Based on the number of the aromatic rings of the compound, polycyclic aromatic hydrocarbons are classified into two categories; low molecular weight (LMW) PAHs composed of two or three aromatic rings such as naphthalene, phenanthrene, and fluorene and high molecular weight (HMW) PAHs with more than three aromatic rings such as benzo[a]pyrene, chrysene, and fluoranthene (53, 69, 71-76). The hydrophobic nature of PAHs is notably correlated with the molecular weight of the compounds where high molecular weight PAHs are more hydrophobic and persistent in the environment than low molecular weight PAHs (76, 77). Polycyclic aromatic hydrocarbons are of considerable concern for environmental health due to their high recalcitrance and stability and hydrophobicity properties (78). They have also been reported for their adverse consequences on human health for their significant toxic properties as mutagenic and carcinogenic agents. (39, 53, 79, 80). As they enter the human body, they form hydroxylated products, particularly in the liver, that can easily interfere with the DNA

leading to serious diseases (81). High molecular weight (HMW) PAHs, such as pyrene, have been recognized as the most dangerous environmental contaminants for their high risk on human and environmental health and therefore have been identified as environmental priority pollutants by the U.S. Environmental Protection Agency (73, 75, 82, 83).

A number of bacterial strains that can cometabolize or grow on dibenzo-*p*-dioxin and dibenzofuran have previously been isolated and identified as *Pseudomonas*, *Rhodococcus, Terrabacter,* and *Sphingomonas* species (45, 84-86). Species belonging to the sphingomonad group have been identified as the best-isolated aromatic compound degrading bacteria (82). The biodegradation of PAHs is affected by a number of important factors such as the long-term persistence, low water solubility, and the low bioavailability of these compounds in contaminated environments (63, 73, 87). The persistence of PAHs may also be affected by the number and the chemical structure of the aromatic nuclei of each class of the PAHs in addition to the number and the arrangement of any halogens on the aromatic rings (88, 89). For instance, low molecular-weight polyaromatic hydrocarbon, such as naphthalene, are considered more susceptible to biodegradation than high molecular weight polyaromatic hydrocarbons (39, 66, 90). Interestingly, numerous enzymatic systems have evolved in different bacterial communities and several genes encoding dioxygenases that initiate the degradation of the diverse hydrocarbon compounds have been identified using degenerate primers or specific probes (78, 89, 91, 92). Some bacterial species, in addition, share similar initial dioxygenase systems or lower pathway enzymes in order to activate the degradation of the PAH. The presence of high similarity catabolic genes between different xenobiotic

degrading bacteria suggest that these genes have been acquired by the mean of horizontal gene transfer (88).

1.3. DIOXIN AND DIOXIN-LIKE COMPOUNDS:

Dioxins are a large family of 210 different congeners of chemically and structurally related polychlorinated aromatic compounds known for their stability, low bioavailability and highly recalcitrant properties. Chlorinated dioxins are commonly composed of two main groups, the polychlorinated dibenzo-*p*-dioxins consisting of 75 congeners and the polychlorinated dibenzofurans consisting of 135 congeners collectively referred as dioxin-like compounds (93).
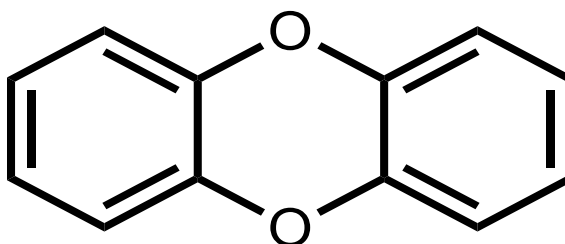
Figure 1. Skeletal structure of dibenzo-*p*-dioxin

Dioxin and dioxin-like compounds are formed and released in large quantities to the environment as a consequence of human and ecological activities and industrial events such as incomplete combustion of organic substances containing chlorine, manufacturing of herbicides and pesticides containing chlorine, paper and pulp bleaching, and incineration of municipal and industrial waste (62, 94, 95). The first industrial release of dioxin pollution to the environment was in 1982 after the Seveso accident in Italy (96, 97). The chemical structure of dibenzo-*p*-dioxin is composed of two benzene rings connected by two oxygen bridges. Dioxin and dioxin like compounds are persistent and

chemically stable contaminants that are well known for their adverse impacts and the serious consequences on environmental and public health (85, 98).

Dibenzo-*p*-dioxin is regarded as a carcinogenic and mutagenic agent and is a main cause of several inflammation cases in human such as chloracne syndrome and chronic skin inflammation (99, 100). Exposure to dioxins can also cause a number of other serious problems such as hepatoxicity, endocrine disorders, reproductive and neuro system disease, and respiratory problems (58, 97, 101, 102). The toxicity of dioxin is mainly due to the number and positions of chlorine atoms attached to the molecule. For instance, the dioxin molecule becomes highly toxic when the chlorine atoms are attached to the carbon atoms at the 2nd, 3rd, 7th, and 8th positions forming a compound called 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most recalcitrant compound found in nature. The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is known as the most potent dioxin congener and that poses a lethal effect on animal and human health and the environment (103-106).

Because of their high recalcitrance, low solubility in water, and the high stability to biological degradation, dioxins have been reported to be the most dangerous environmental pollutants to human and environmental health and their removal from contaminated sites has become a challenge to bioremediation and bioaugmentation technologies. The degradation of dioxins occurs at very low rates in the environment. However, in the last few decades, a number of bacterial isolates, mainly *Pseudomonas*, *Burkholderia*, *Arthrobacter*, *Rhodococcus,* and *Sphingomonas* species, have been reported for their ability to break down the carbon structure of dioxin and other polycyclic aromatic hydrocarbons through a process called bioremediation (107).

The lipophilic properties and the limited solubility of dioxins in water cause these compounds to be easily accumulated and stored in the food chain (Chang, 2008, Cho and Kim, 2001, Takada et al, 1996). The highly toxic effect of dioxin compounds is associated with the binding of the dioxin molecule to a cytoplasmic receptor protein called the aryl hydrocarbon (Ah) receptor; a ligand-induced transcription factor that belongs to the helix-loop-helix (bHLH) superfamily of environmental response proteins (108-112). The extremely toxic dioxin congeners such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) bind with high affinity to the cytoplasmic Ah receptor leading to serious health effects (113-115). In addition, the planar structure enables this compound to form a complex with the AHR receptor that interfere with the regulation of transcription in the cell causing serious effects on human health such as cancer, immune system problems, and developmental and reproductive diseases (116).

1.4. BIOREMEDIATION

Microorganisms including bacteria and fungi play an important role in breaking down the highly toxic compounds in contaminated sites into harmless forms throughout an effective process called bioremediation (117). These microorganisms have the potential to grow and utilize the toxic environmental compounds as the sole source of carbon and energy (40, 118). Bioremediation is known as a powerful approach for the removal and detoxification of organic contaminants in the environment including polycyclic aromatic hydrocarbons (43, 119). It has been effectively used as an alternative treatment to traditional methods including physical-chemical strategies of dioxin removal from polluted sites. (69, 120). The process of bioremediation can be carried out using two important approaches, bioaugmentation and biostimulation (121). While bioaugmentation

involves the introduction of bacterial strains to the contaminated sites to stimulate pollutant degradation, biostimulation requires providing optimal conditions including temperature, nutrients, and aeration in order to promote bioremediation capabilities of microorganisms already present at the contaminated site and often involves using favorable electrons donors and acceptors that simulate the metabolic activities of the microorganisms in the contaminated environments (76, 117). Aerobic microbial degradation has been a subject of interest to the bioremediation field, in particular for the remediation of contaminated sites with the highly recalcitrant compounds dibenzo-*p*-dioxin and dibenzofuran as well as other contaminants such as pharmaceuticals and organic solvents (119, 122). In addition, a number of bacterial strains are known to harbor the catabolic pathways and to completely mineralize the toxic compounds into less harmful structure. In recent years, research has focused on isolating potential bacterial strains that can be utilized in bioremediation (123). Furthermore, microorganisms have evolved new metabolic pathways that enable them to utilize a wide range of polycyclic aromatic hydrocarbons as a source of carbon (89). Microorganisms utilize three main strategies in order to break down the recalcitrant structure of highly toxic compounds. These include oxidative degradation using dioxygenases in aerobic bacteria, anaerobically via reductive dehalogenation, and fungal oxidation utilizing cytochrome P_{450} enzymes. Because of the hazardous impacts of xenobiotic compounds on the environment, bacterial degradation has been the focus of much research over the last 100 plus years. A number of bacterial strains have been isolated for their remarkable ability to reduce the concentration and even completely eliminate environmental organic pollutants. Among these bacteria, *S. wittichii* RW1 has been shown to be a promising

microorganism for biodegradation and for the removal of dioxin from the contaminated

sites.

CHAPTER 2: MATERIALS AND METHODS

2.1 BACTERIAL STRAINS, PLASMIDS, AND CULTURE CONDITIONS

Sphingomonas wittichii RW1 (48) and *Sphingobium yanoikuyae* B1 (124) were cultivated aerobically at 30°C on Luria-Bertani (LB) agar medium or mineral salts basal medium (MSB) (125) with either 10 mM phenylalanine or 10 mM succinate. *E. coli* strains were grown on LB agar medium at 37°C supplemented with the appropriate antibiotics. *E. coli* DH5α was used as a host strain for standard cloning and transformation. Growth media were supplemented with different antibiotics including kanamycin (Km) (100 µg ml^{-1}), ampicillin (Amp) (50 µg ml^{-1}), tetracycline Tc (15 µg ml^{-1}) or gentamycin (Gm) (25 µg ml^{-1}) in order to screen for the resultant clones or to select for transformants, transconjugants, and knockout mutants.

2.2. CHEMICALS AND REAGENTS

Most chemicals used in this study were purchased from Sigma-Aldrich or Baker and were of the highest grade available. Luria-Bertani (LB) medium was obtained from Becton, Dickinson and Company (Sparks, MD, USA). Dibenzo [b,e], [1, 4] Dioxin and Dibenzofuran were purchased from Tokyo, Japan (Toshima, Kita-Ku, Tokyo, Japan). Difco^{TM} Noble agar was obtained from Becton, Dickinson and Company (Sparks, MD, USA) and used at a concentration 2% to solidify the mineral salts basal medium (MSB) Succinic acid, sodium salicylate, and L-phenylalanine were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Dibenzo-*p*-dioxin, dibenzofuran, L-phenylalanine, salicylate, and succinate were added to the mineral salts basal medium (MSB) at a final concentration of 3 mM, 3 mM, 10 mM, 5 mM, and 10 mM, respectively. For growth curve experiments, the carbon sources dibenzo-*p*-dioxin or dibenzofuran were

dissolved in acetone and left in the hood for 6 hours to evaporate the acetone. 50 ml of

MSB was then added and the flasks sonicated for 5 minutes to allow the dibenzo-*p*-dioxin

or dibenzofuran crystals to disperse. For MSB agar medium, dibenzo-*p*-dioxin and

dibenzofuran were provided as crystals in the petri dish lids.

2.3. PLASMID DNA EXTRACTION AND MOLECULAR GENETICS METHODS

Ligation reaction using T4 DNA ligase and restriction enzyme digestions with

CutSmart buffer were set up following the instructions provided by the supplier (New

England BioLabs Inc). Transformation and agarose gel electrophoresis were performed

using standard techniques. Agarose gel electrophoresis was carried out in Tris (40 mM)

acetate (20 mM) and EDTA buffer (2 mM). Total genomic DNA was extracted using the

UltraClean ® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA)

according to the manufacturer's recommendations. Plasmids were isolated with the

NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, Germany) according to

the manufacturer's instruction. DNA fragments were analyzed by electrophoresis on 0.8%

agarose gels and purified by gel extraction using the Geneclean® III Kit (MP

Biomedicals, LLC, IIIkrich, France). The kanamycin, kanamycin3, and gentamycin

antibiotic cassettes (126, 127) were retrieved from the plasmids p34S-km, p34S-km3, and

p34S-gm, respectively, using the primers outlined in Supplemental Table 2.

2.4. PRIMER DESIGN

All primers were designed using the Lasergene SeqBuilder software (version 10,

DNASTAR, Madison, WI, USA). The primer sequences and plasmids used in this study

are listed in Supplemental Table 1 and 2.

2.5. PCR CONDITIONS

For PCR reactions, the Phusion® High-Fidelity PCR Kit (New England BioLabs Inc) and the ReadyMix™ Taq PCR Reaction Mix (Sigma, St. Louis, MO, USA) were utilized following the manufacturer's directions. PCR purification products were purified from PCR reactions using Geneclean® III Kit (MP Biomedicals, LLC, IIIkrich, France. All sequencing was performed by Genewiz (South Plainfield, NJ, USA). The thermal cycling conditions for Phusion® High-Fidelity PCR reactions were as follows: 98°C for 30 sec, 98°C for 10 sec, 72°C for 30 sec, 72°C for 30 sec, 72°C for 7 min and 4°C hold.

2.6. OVERLAP EXTENSION PCR

The PCR reactions were carried out using Phusion ® High-Fidelity PCR Kit (New England BioLabs Inc). The thermal cycling program for overlap extension PCR is as follows: the first 10 cycles were run without adding primers using a program with 94°C for 3 minutes, 94°C for 30 seconds, 60°C for 1.5 minutes, and 72°C for 2.5 minutes. Next, the forward primer 5Z-oriR-lacZ and the reverse primer TnMod-oriT3 were added and 25 cycles were run using the program 94°C for 5 minutes, 94°C for 30 seconds, 72°C for 2 minutes, and 72°C for 3 minutes.

2.7. TRIPARENTAL CONJUGATION

The bacterial triparental conjugation was set up using the "gob" method. In this method, the three bacterial strains (the donor, the recipient, and the helper strain) were initially grown overnight on LB agar plates supplemented with the appropriate antibiotic. Each strain was suspended in 500 µl LB broth, pelleted together by centrifugation at 3000 rpm for 3 min, and resuspended in 50 µl of LB broth. The mixture was placed as a spot on an LB plate and incubated overnight at 30°C.

2.8. GENE KNOCKOUT

2.8.1. *DxnA1A2* **gene knockout**

2.8.1.1. Construction of plasmid and ΔdxnA1A2::Km mutant

To generate the ΔdxnA1A2::Km knockout mutant, total microbial DNA was extracted from the wild type strain *S. wittichii* RW1 using UltraClean® Microbial DNA extraction kit (Mobio, Carlsbad, CA). The *dxnA1A2* genes encoding the angular dioxin dioxygenase were amplified from genomic DNA of *S. wittichii* RW1 by PCR with primers dxn-A1-ko-f1 and dxnA1-ko-r1 (Table 1) using Phusion® High-Fidelity PCR Kit (New England BioLabs Inc). The resultant amplicon (2.7 kb) was purified from the PCR reaction using the Geneclean® III Kit (MP Biomedicals, LLC, IIIkrich, France), digested with *Hin*dIII and *Xba*I, cloned into the corresponding sites of the unstable cloning vector pRK415, and transformed into competent *E. coli* DH5α cells. A 1.3 kb gene sequence encoding a kanamycin resistance cassette excised from p34S-Km plasmid was subcloned into the *Pst*I site of the *dxnA1A2* genes. The resultant construct was introduced into *S. wittichii* RW1 by triparental conjugation using pRK2013 as the helper plasmid. The transconjugant carrying the donor vector was confirmed by PCR and was subjected to a knockout by inoculating 5 ml of fresh LB broth medium with a single colony. A 100 μl volume was subcultured every 24 hours to another 5 ml of LB broth medium. After the 8th to 10th subculture transfer, the culture was diluted and 100 μl from the 10^{-2} and 10^{-3} dilution was spread on LB agar plates supplemented with the appropriate antibiotic. The resultant colonies were screened on LB Tc plate and LB Km plate for the loss of the unstable vector as a result of homologous recombination of the kanamycin gene into the chromosome. Next, the knockout mutant was tested for loss of the ability to utilize

dibenzofuran and dibenzo-*p*-dioxin as the sole carbon source. To confirm our result, a

growth curve was performed to measure the growth rate of the knockout mutant in

comparison with the wild type strain.
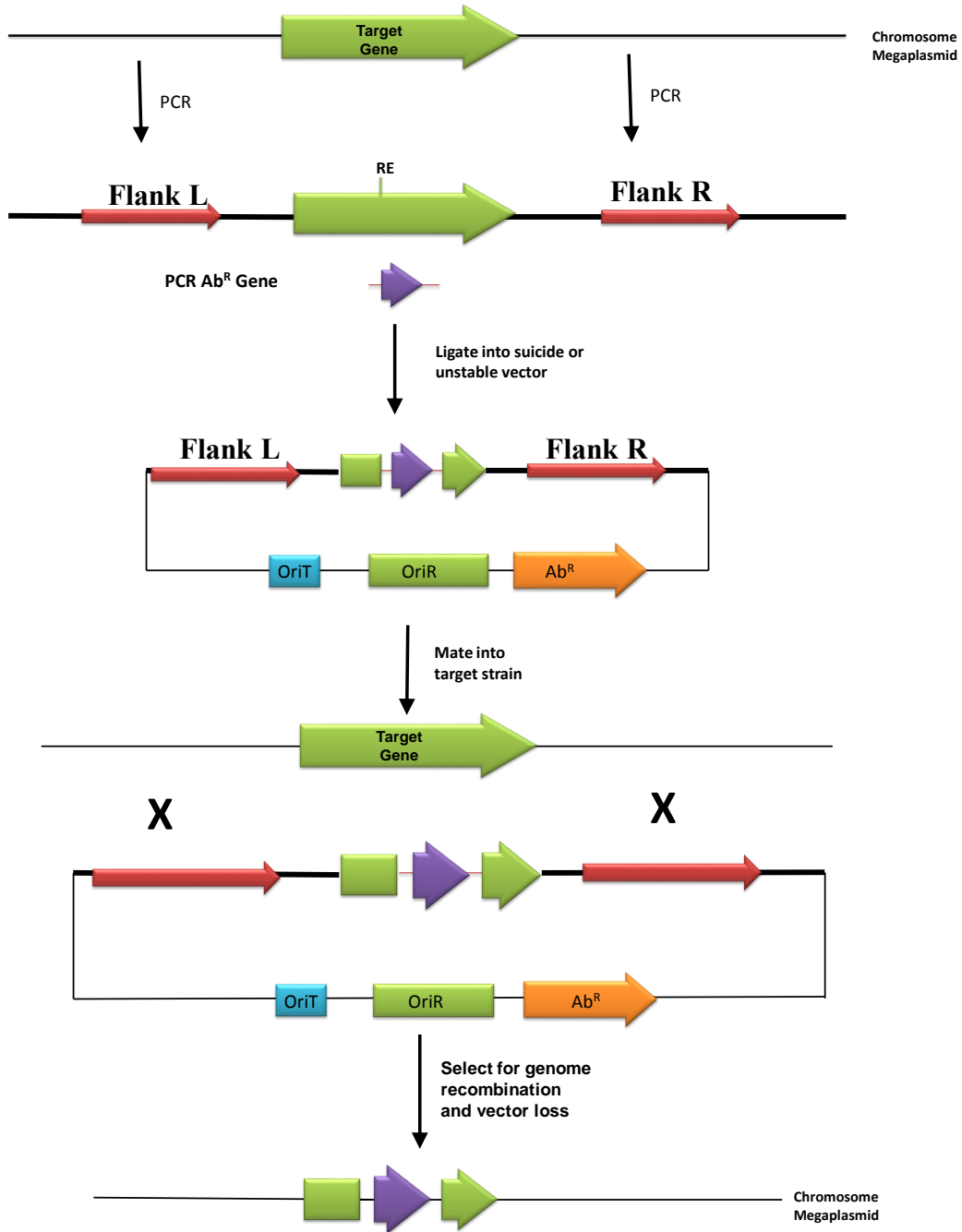
Figure 2. Schematic representation of gene knockout procedure

2.8.1.2. Complementation

To ensure the activity of the intact *dxnA1A2* gene, the functional *dxnA1A2* gene was PCR amplified with primers dxn-A1-ko-f1 and dxnA1-ko-r1 containing *Hin*dII and *Xba*I sites respectively, cloned into the cloning vector pRK415 and introduced into the *dxnA1A2* knockout mutant by triparental conjugation. The resultant transconjugant was tested for ability to grow on MSB supplemented with either dibenzo-*p*-dioxin or dibenzofuran as the sole source of carbon and energy at 30°C.

2.8.2. *redA2* gene knockout

In order to obtain a knockout mutant of the redA2 gene, the $5^{'}$ upstream fragment was isolated from genomic DNA of *S. wittichii* RW1 by PCR with primers RedA2-f with *Sac*I site and RedA2-mr with *Kpn*I site, forward and reverse primer, respectively (Table 1) using Phusion® High-Fidelity PCR Kit (New England BioLabs Inc). The resultant PCR product was cloned into the pGEM®-T Easy Vector (Promega Cooperation, Madison, WI, USA) by TA cloning method using 2X rapid ligation buffer. The ligation reaction was incubated at 4°C overnight. After the incubation period, 1 µl of the ligation reaction was transformed into competent DH5α cells and the clones were selected on LB/Amp/X-gal agar medium. The clone with the right size was verified by sequencing. Next, the $3^{'}$ downstream fragment was PCR amplified with the primers RedA2-mf with *Kpn*I site and RedA2-r with *Bam*HI site as the forward and reverse primers, respectively. The amplified DNA fragment was cloned into the pGEM®-T Easy Vector by TA cloning. The ligation reaction was transformed into *E. coli* DH5alpha cells and clones were selected on LB/Amp/X-gal agar medium. The clone with the right size was then verified by sequencing. The upstream and downstream flanking areas were digested from

the pGEM®-T Easy vector using the restriction endonucleases *Sac*I/*Kpn*I and

*Kpn*I/*Bam*HI, respectively. The resultant 1.3 kb upstream fragment and 1.0 kb

downstream fragment were gel purified and ligated into plasmid pRK415 at the

corresponding restriction sites. Next, a 1.3 kb fragment containing the kanamycin cassette

was retrieved from plasmid p34s-km3 and cloned at the *Kpn*I site. To confirm the gene

knockout mutant of *redA2*, the knockout mutant was PCR amplified with the primers

km3-R and RedA2-R. The gene knockout mutant was verified using the primers RedA2-

R2 and km3-R because the construct and the mutant are with the same size (3 kb).

2.8.3. *redA1* gene knockout

To generate a knockout mutant of the *redA1* gene, the upstream flanking region

(960 bp) of the *redA1* gene was amplified by PCR using genomic DNA from *S. wittichii*

RW1 as a template and the primers RedA1-MR and RedA1-F1 that introduced *Eco*RI and

*Sac*I restriction sites at the 5' and 3' ends of the DNA fragment, respectively. The

amplified DNA product of the predicted size was cloned into the pGEM-T easy vector by

the TA cloning method. The resultant clone with the right size of the insert was digested

with *Eco*RI and *Sac*I. The digested upstream fragment was then excised and purified

from the 0.8% agarose gel. The downstream fragment (1 kb) was PCR amplified with

primers RedA1-MF and RedA1-R which introduce a *Bam*HI site and a *Sac*I site at the

two ends of the DNA fragment. The PCR amplification product was purified and cloned

into the pGEM-T easy vector using the pGEM-T Easy cloning kit. The resultant clone

was verified by sequencing and then digested with *Bam*HI and *Sac*I and run on agarose

gel. The *Bam*HI/*Sac*I fragment was excised and purified from the gel. The digested

product of both the upstream and downstream fragments were then cloned into the

pRK415 vector at the corresponding site to yield pRK415/RedA1. Next, a gentamycin

cassette (850 bp) was digested with *Sac*I, purified from the gel, and cloned into

pRK415/RedA1 at the corresponding site. The final construct was then moved into *S.*

wittichii RW1 via triparental mating and a knockout constructed and tested as explained

above.

2.8.4 Complementation

In order to ensure that the specified gene has the correct function, the DNA of the

intact gene was PCR amplified from the wild-type strain with primers RedA1-FC1 and

RedA1-RC1 for RedA1 gene complement and the primers RedA2-FC1 and RedA2-RC1

for the RedA2 complement. The resulting PCR product was then cloned into the pGEM-

T easy vector by means of TA cloning. After confirming the correct gene by sequencing,

the gene was retrieved from the pGEM-T easy vector and re-cloned into plasmid

pRK415. The final construct was then moved into the double knockout mutant bacterium

by means of triparental conjugation.

2.8.5. *fdx3* gene knockout

To obtain the *fdx3* gene knockout, a 924 bp upstream region of the *fdx3* gene was

PCR amplified with primers Fdx3-f with a *Hin*dIII site and Fdx3-mr with a *Xba*I site

using Phusion® High-Fidelity PCR Kit (New England BioLabs Inc).   The PCR product

of the upstream fragment was purified and cloned into the pGEM®-T Easy Vector

(Promega, Madison, WI, USA). Next, a 1,030-nucleotide downstream region of the *fdx3*

gene was PCR amplified with primers Fdx3-mf with a *Xba*I site and Fdx3-r with a *Kpn*I

site, The PCR product was purified and cloned into the pGEM®-T Easy Vector by the

TA cloning method using 2X Rapid ligation Buffer. The ligation reaction was incubated

at 4°C overnight and 3 µl was transformed into DH5α. Cells were then plated on LB agar

plates with ampicillin and X-gal and incubated at 37°C overnight. A clone with the correct size was selected and verified by sequencing. The upstream region of the gene was excised from the pGEM-T vector with *Hin*dII and *Xba*I and the downstream region of the gene was excised from the pGEM-T vector with *Xba*I and *Kpn*I and both the upstream and downstream fragments were cloned into the pRK415 vector. Next, the km3 cassette (1 kb) was excised from p34S-Km3 plasmid and cloned into the *Xba*I site of the *fdx3* gene. The resultant construct was moved into *S. wittichii* strain RW1 via triparental mating and the knockout mutant constructed as described above.

2.8.6. *fdx1* gene knockout

To generate the *fdx1* gene knockout, a 1,084 bp fragment comprising the 5' upstream flanking region of the *fdx1* gene was PCR amplified from genomic DNA of *S. wittichii* RW1 with primers Fdx1-F1 with an added *Eco*RI site and Fdx1-mr with an added *Sac*I site using the Phusion® High-Fidelity PCR Kit (New England BioLabs Inc). The resulting PCR fragment was then cloned into the pGEM®-T Easy Vector (Promega, Madison, WI, USA). The clones were selected on LB medium with kanamycin and X-gal. The correct clone was digested with *Eco*RI and *Sac*I and the correct DNA fragment excised from the gel. Next, the 3' downstream flanking region (1,188 bp) of the *fdx1* gene was PCR amplified with primers Fdx1-mf with an added *Sac*I site and Fdx1-R with an added *Bam*HI site, The PCR product was purified and cloned into the pGEM®-T Easy Vector by the TA cloning method using 2X Rapid ligation Buffer. The ligation reaction was incubated at 4°C overnight and 3 µl was transformed into competent *E. coli* DH5α cells. Cells were then plated on LB agar plates with ampicillin and X-gal and incubated at 37°C overnight. A clone with the correct size was selected and verified by

sequencing. The upstream region of the gene was excised from the pGEM-T vector with *Eco*RI and *Sac*I and the downstream region of the gene was excised from the pGEM-T vector with *Sac*I and *Bam*HI, and both the upstream and downstream fragments were cloned into pRK415 digested with *Eco*RI and *Bam*HI. Next, the gentamycin cassette (1 kb) was excised from the p34S-Gm plasmid and cloned into the *Sac*I site. The resultant construct was moved into *S. wittichii* strain RW1 via triparental mating and a knockout strain constructed as described above.

2.8.7. Complementation

In order to verify the function of the target genes that are responsible for the initial dioxygenolytic attack of dibenzo-*p*-dioxin and dibenzofuran, the *fdx1* and *fdx3* genes encoding the two ferredoxins were PCR amplified with primers Fdx1-FC1 AAGCTTctgccagtaaaaatttgcattgg and Fdx1-RC1; GGATCC gatttcaaatctacgctgtgg and Fdx3-FC1 CTGCAGgcaccatctctcggatgggag and Fdx3-RC1 GGATCC ggataactttggaggatcg, respectively. The PCR products were then cloned into the broad host range vector pRK415 at the corresponding sites. The resultant clones were then confirmed by sequencing and mobilized into the mutant strain via triparental mating. The resulting transconjugant was then tested for the ability to grow on MSB supplemented with dibenzo-*p*-dioxin or dibenzofuran.

2.9. CONSTRUCTION OF A NEW SHUTTLE VECTOR PSEZ-B1RP

The construction of a new plasmid vector requires the presence of a number of important putative parts including an origin of replication oriR associated with partitioning genes, an origin of transfer oriT, and an antibiotic resistance marker such as Tc^R. The new *Sphingomonas* plasmid vector was constructed as follows. Genomic DNA

of *S. yanoikuyae* B1 was extracted using the UltraClean® Microbial DNA (MO BIO,

Carlsbad, CA). The *Xho*I-*Bgl*II 2.4 kb region containing *parA-repA-oriR* was PCR

amplified using the Phusion ® High-Fidelity PCR Kit (New England BioLabs Inc) from

the plasmid pKG2, a cryptic plasmid in *S. yanoikuyae* B1 with primers par_rep_5 and

par_rep_3. The PCR amplification product was purified from solution using the

Geneclean® III kit and incorporated into the pGEM-T Easy vector by TA cloning. The

transformed DH5α colonies were plated on LB agar supplemented with ampicillin and

Xgal. The desired clone was then confirmed by sequencing. A 2.0 kb fragment with the

tetA-tetR genes for tetracycline resistance was PCR amplified from pRK415 using the

415_Tc_5 and 415_Tc_3 primers containing the restriction sites *Bgl*II and *Kpn*I,

respectively. The PCR product was purified using the Geneclean® III Kit (MP

Biomedicals, LLC, IIIkrich, France) and cloned into the pGEM-T easy vector. The

ligation reaction was transformed into competent DH5α cells and the correct clone

selected on LB-Amp-Xgal agar plates. Clones with the correct insert size were then

verified by sequencing. The *E. coli* plasmid OriR region was isolated from pGEM5Z(f-)

using the Phusion® High-Fidelity PCR Kit (New England BioLabs Inc) with primers 5Z-

oriR-lacZ-5 (forward primer) and 5Z-OriR-lacZ-3 (reverse primer). The OriT fragment

was isolated from plasmid pTnMod-Okm3 using the Phusion® High-Fidelity PCR Kit

(New England BioLabs Inc) with primers TnMod-oriT-5 (forward primer) and TnMod-

oriT-3 (reverse primer). After PCR, the OriR fragment and the OriT fragment were run

on a 0.8% agarose gel and excised and purified using the Geneclean® III Kit (MP

Biomedicals, LLC, IIIkrich, France). The purified products of both PCR fragments were

used as a template for the overlap extension PCR. The first 10 cycles were run without

primers and 25 cycles were run with the primers 5Z-oriR-lacZ-5 (forward primer) and

TnMod-oriT-3 (reverse primer) using the Phusion® High-Fidelity PCR Kit (New

England Biolabs. Inc). The amplified fragment was run on 0.8% agarose gel and purified

from the gel using Geneclean® III Kit (MP Biomedicals, LLC, IIIkrich, France). The

purified 1.8 kb fragment with the pMB1 *oriR*, *lacZ* (MCS), and RK2 *oriT* was

sequentially cut with *Kpn*I and then with *Xho*I. The resulting three PCR fragments were

ligated together using the respective restriction enzyme cutting sites as shown in Figure 3.

The final construct was verified by complete sequencing.
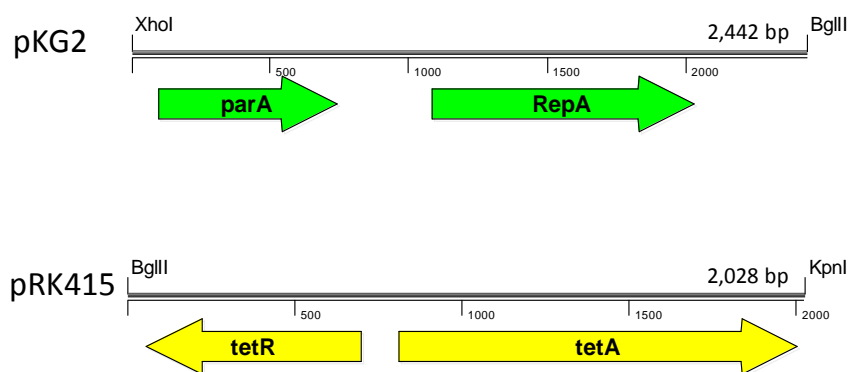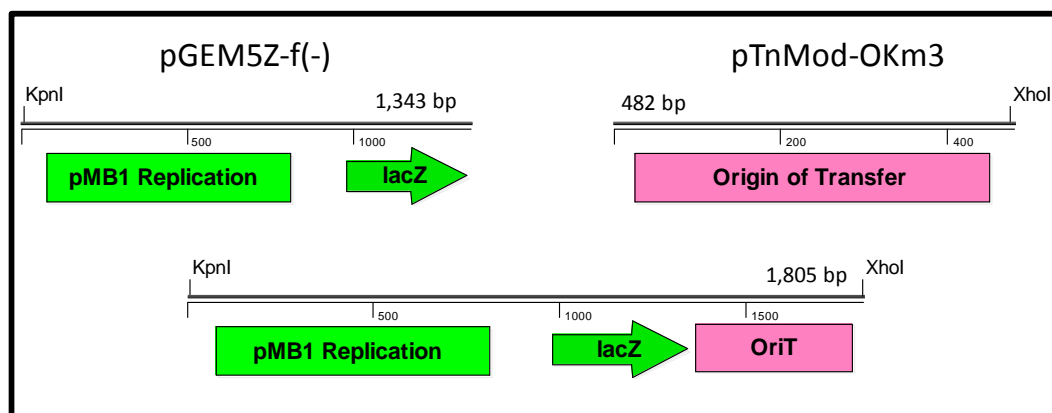
Figure 3: Construction of a new Sphingomonad plasmid vector

2.9.1. Deletion of the partitioning region parA from the new vector pSEZ-B1RP

To remove the *parA-repA-oriR* which encodes for the partitioning system of the new cloning vector pSEZ_B1RP, the plasmid DNA was isolated from the host *E. coli* DH5α, digested with *Bgl*II and *Xho*I and run on a 0.8% agarose gel. The plasmid backbone (minus the *parA-repA-oriR* region) of 3.8 kb was excised and purified from the gel. The *repA* fragment (1.7 kb) was isolated from pKG2 plasmid of strain B1 with primers end-par introducing a *Xho*I site and par-rep3 introducing a *Bgl*II site using Readymix Taq. The 1.7 kb PCR fragment was purified and cloned into the pGEM-T-easy vector by TA cloning. After verifying by sequencing, the *repA* fragment was removed by digestion with *Xho*I and *Bgl*II. The gel-purified *repA* fragment was then ligated with the 3.8 kb vector backbone. The ligation reaction was incubated at 16℃ overnight, transformed into DH5α, and plated on LB agar with tertracycline. The resultant vector was then cut with *Bgl*II and *Xho*I which yields two fragments (3.7 kb and 1.7 kb) to verify the correct size of the resulting vector.

2.9.2. Examine the stability of the new vector pSEZ-B1RP and pSEZ-B1R in *Sphingomonas yanoikuyae* strain B1

To assess the stability of the new vector pSEZ-B1RP in *S. yanoikuyae* B1, the plasmid vector was introduced into the recipient cells of B1 by triparental conjugation using the "Gob method" procedure. In this method, the recipient strain B1 was grown on an LB agar plate at 30°C overnight. *E. coli* HB101 containing the helper plasmid pRK2013 was grown on an LB Kanamycin plate overnight at 37°C and *E. coli* DH5α containing the donor plasmid was grown on LB Tetracycline overnight at 37°C. A loop full of the bacterial cells of the recipient, the donor, and the helper was each

suspended in 500 µl of LB broth medium. After centrifugation at 3,000 rpm for 3 minutes, the supernatant was discarded and the pellets of the helper strain and the donor strain were suspended in 200 µl LB broth. Then, 25 µl of the helper and 25 µl of the donor were added to the pellet of the recipient strain, mixed together, and the suspension was plated on LB agar medium and incubated at 30℃ overnight. After incubation, the cells were suspended in 1 ml of LB broth and serial dilutions were made. Then, 100 µl from the 10^{-3} and 10^{-4} dilutions were spread on MSB medium supplemented with succinate for selection. The transconjugant carrying the new plasmid was confirmed by PCR with primers lacZ mapF and RepA mapR (Table 2). In order to obtain a pKG2 free strain, the resulting transconjugant was grown in 5 ml LB broth with tetracycline and incubated at 30°C overnight. After two subculture transfers in LB broth with tetracycline, individual colonies were selected and assessed for the loss of the pKG2 plasmid using PCR with primers BlankmapF (forward primer) and RepAmapR (reverse primer). Then, one single colony that exhibited loss of the cryptic plasmid pKG2 was used to test the stability of the new cloning vector. The colony was first grown on LB tetracycline plates and incubated at 30°C overnight. The growing cells were suspended in 350 µl LB broth and 100 µl was transferred to 3 tubes of 5 ml LB broth and incubated at 30°C overnight.

2.9.3. Stability of the new vector pSEZ-B1RP in *Sphingomonas wittichii* RW1

To examine the stability of the new cloning vector pSEZ-B1RP in *S. wittichii* RW1, the cloning vector was introduced into RW1 via triparental mating using pRK2013 as a helper plasmid. The resulting transconjugant was grown on MSB succinate supplemented with tetracycline and incubated at 30℃ for two days. Then, 5 ml of LB broth was inoculated with the growing bacterial cells. 50 µl was transferred from the LB

broth tube to another 5 mls of LB broth and incubated at 30℃ overnight. This step was repeated for 6 days. Next, serial dilutions were made from each tube and 100 µl from 10^{-3} and 10^{-4} dilutions were plated on LB agar and LB tetracycline plates. On the first day, after 24 hours incubation at 30℃, the numbers of the colonies were almost the same on both the LB agar and LB agar with tetracycline. However, the numbers of colonies on LB tetracycline plates decreased highly on the second days and there were no growth at all on the LB tetracycline plates on the third day.

2.10. ELIMINATION OF THE MEGAPLASMID

The new plasmid vector pSEZ_RW1RP (8,177 bp) was developed from the previously constructed vector pSEZ-B1RP. The origin of replication of pSEZ-B1RP was removed and replaced by the pSWIT02 oriR obtained from the total DNA of *S. wittichii* RW1. First, the plasmid pSEZ-B1RP was digested with the restriction endonucleases *Bgl*II and *Xho*I. A 3.4 kb fragment containing the origin of transfer (oriT) gene, the lacZ gene and the tetracycline resistance genes was retrieved from the plasmid pSEZ-B1RP (digested and purified from the gel). Next, the origin of replication of plasmid pSWIT02 along with the genes for partitioning were obtained from genomic DNA of *S. wittichii* RW1 by PCR amplification with the forward primer parB-F containing a *Bgl*II site and the reverse primer Rep-R containing a *Xho*I site. The resulting 4.3 kb fragment was purified from the PCR reaction and ligated into the pGEM-T Easy vector. The clone with the correct insert size was selected based on the blue white screening method and confirmed by sequencing. The 4.3 kb fragment containing the *RepA-parB* genes was retrieved from the pGEM-T Easy vector with the restriction sites *Bgl*II and *Xho*I. After purification, the two fragments (the 3.8 kb region of plasmid pSEZ-B1RP and the 4.3 kb

region with the *RepA-ParB* region) were ligated together with T4 ligase. The construct

was transformed into *E. coli* DH5α and the construct verified by restriction mapping.

The construct, designated pSEZ_RW1RP was then transferred to *S. wittichii* RW1 by

triparental mating with tetracycline selection. RW1 containing pSEZ_RW1RP was

subcultured several times in MSB liquid medium supplemented with 10 mM

phenylalanine. Selection of cured RW1 was carried out by screening colonies on MSB

agar plates supplemented with dibenzo-*p*-dioxin or dibenzofuran crystals. To verify the

loss of the megaplasmid pSWIT02, the wild-type strain *S. wittichii* RW1 and the cured *S.*

wittichii RW1 were analyzed by PCR with the primers RepA-F,

GGAGATCTGGTTGCATCAGTGGCCATAGG, as a forward primer, and RepA-R2,

TCAACGTGAACGGCTCGGAG, as a reverse primer, and RepA-F as a forward primer

and RepA-R3, CACCTTCATCACCAGCAACC, as a reverse primer. The wild type

strain gives a 1.9 kb band from RepA-F and RepA-R2 and a 2.6 kb fragment from RepA-

F and RepA-R3.

2.11. REMOVAL OF THE NEW VECTOR PSEZ_RW1RP FROM PSWT02 FREE RW1

The new plasmid vector pSEZ_RW1RP was removed from the cured RW1 as

follows. The cured RW1 strain was grown in 5 ml of LB broth and incubated at 30°C

overnight. Serial dilutions were then made and cells from the 10^{-3} and 10^{-4} dilutions were

plated on LB agar medium. To test for the curing of the vector pSEZ_RW1RP, 100 single

colonies were transferred to LB tetracycline medium and LB medium without antibiotic.

The colonies that are sensitive to tetracycline were selected from LB agar medium and

confirmed by PCR for the loss of the vector pSEZ_RW1RP.

2.12. STABILITY OF THE NEW VECTOR PSEZ-PSWIT02 BA02-REPA02 AND PSEZ-PSWIT02 REP-02 IN CURED *S. WITTICHII* RW1

To assess the stability of the new plasmid vector, the two new *Sphingomonas* vectors, pSEZ-pSWIT02 BA02-RepA02 or pSEZ-pSWIT02 RepA02, were introduced into the cured S. *wittichii* RW1 via triparental mating. The resulting transconjugants carrying the new vector pSEZ-pSWIT02 BA02-RepA02 or pSEZ-pSWIT02 Rep-02 were grown on LB agar medium supplemented with tetracycline at 30°C overnight. The grown cells were then suspended into LB broth medium (without selective pressure) and a 100 µl from the suspension was transferred to three tubes each containing 5 ml of LB broth medium and incubated at 30°C overnight. Next, 100 µl was transferred from each tube into another 5 ml of LB broth and incubated at 30°C overnight. Serial dilutions were then made from the first transfer and a 100 µl from the 10^{-3} and 10^{-4} dilutions was spread on LB agar medium without selective pressure and incubated at 30°C for four days. Next, 50 colonies were transferred to LB agar medium supplemented with tetracycline and the number of the sensitive colonies were counted out of 50.
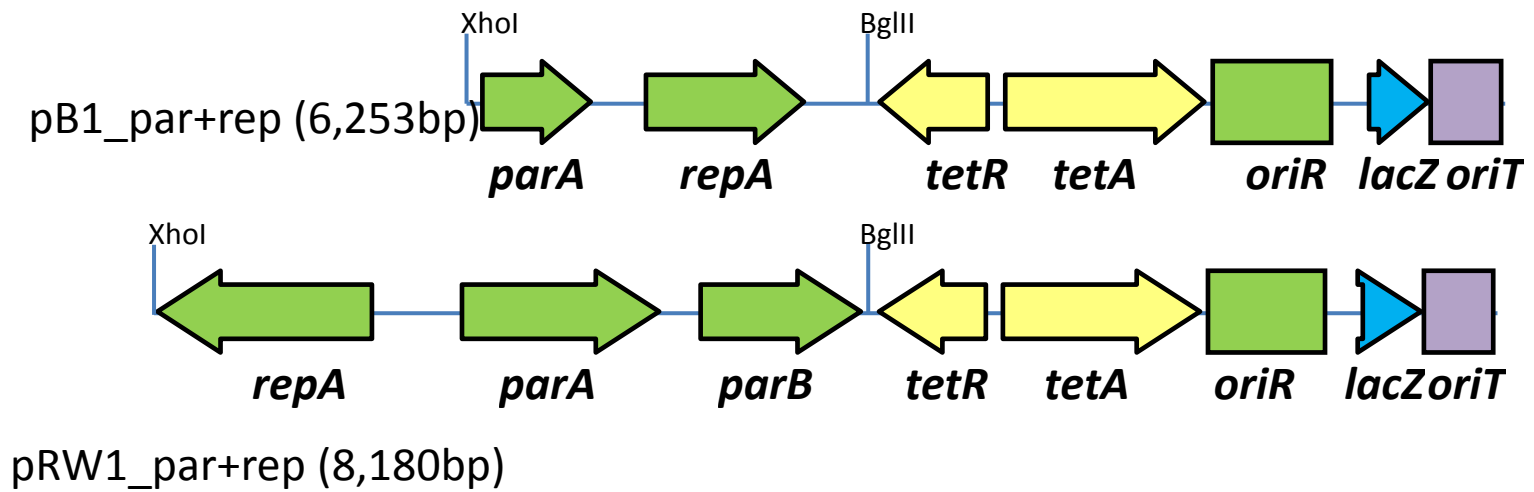
Figure 4. Construction of the pSWIT02-derived plasmid

2.13. GROWTH CURVE

To measure the turbidity of growing cells, the bacterial cells of *S. wittichii* RW1 wild type and the knockout mutants were grown in 5 ml LB broth each with an appropriate antibiotic and incubated at 30°C with agitation overnight. When the OD_{600} reached 1.5 , the growing bacterial cells were transferred to 50 ml of MSB with phenylalanine to a starting OD_{600} of 0.1 and incubated at 30°C for 12 hours. When the optical density reached 1.2 to 1.5 the bacterial cells were then transferred to 50 ml of MSB supplemented with either 3 mM dibenzo-*p*-dioxin or dibenzofuran as the only carbon source with a starting OD_{600} of 0.05. Growth was measured first after 8 hours and then every 3 hours at OD_{600} for the dibenzo-*p*-dioxin culture and first after 6 hours and then every 2 hours at OD_{600} for the dibenzofuran culture.

Table 1: Plasmids and Primers

Strain/Plasmid	Feature	Source/Reference
pTnModOKm3	Km^r	Dennis, J. J., & Zylstra, G. J. (1998).. *Applied and environmental microbiology*, *64*(7), 2710-2715.
DH5α	*supE44 Δ lacU169 (ϕ80 lacZΔ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
pRK415	Tc^r, broad host range vector	N.T. Keen, S. Tamaki, D. Kobayashi, and D. Trollinger. 1998.
pRK2013	Km^r	Knauf VC, Nester EW. Plasmid 8: 45-54, 1982.
S. wittichii RW1	PAH degrading bacterium	Wittich RM, Wilkes H, Sinnwell V, Francke W, Fortnagel P. 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl Environ Microbiol 58:1005-10.
S. yanoikuyae B1	PAH degrading bacterium	Gibson DT, Roberts RL, Wells MC, Kobal VM. 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem Biophys Res Commun 50:211-9.
P34S-km	Km^r	Dennis, J. J., & Zylstra, G. J. (1998). *Applied and environmental microbiology*, *64*(7), 2710-2715.
P34S-km3	Km^r	Dennis, J. J., & Zylstra, G. J. (1998). *Applied and environmental microbiology*, *64*(7), 2710-2715.
P34S-GM	Gm^r	Dennis, J. J., & Zylstra, G. J. (1998). *Applied and environmental microbiology*, *64*(7), 2710-2715.
pGEM T-Easy	Am^r, broad host range cloning vector	Promega

Primer Name	Forward Primer	Reverse Primer	Anneal temperature (°C) using Phusion or ReadyMix
FDX3-FC1 FDX3-RC1	CTGCAGGCACCAT CTCTCGGATGGGA G	GGATCCGGATAAC TTTGGAGGATCG	72°C Phusion 56°C ReadyMix
FDX1-FC1 FDX1-RC1	AAGCTTCTGCCAG TAAAAATTTGCAT TGG	GGATCCGATTTCA AATCTACGCTGTG G	72°C Phusion 55°C ReadyMix
RedA1-FC1 RedA1-RC1	CTGCAGGCATAGA GAAAGCCGGGAG	GGATCCCGCGATG CTACGGTTCCCGC	72°C Phusion 62°C ReadyMix
RedA2-FC1 RedA2-RC1	CTGCAGGCTAAGG AGGGCAGGTTCTG G	GGATCCGGCGTCT CCCTGCTCCAATA G	72°C Phusion 65°C ReadyMix
par_rep_5 par_rep_3	CTCGAGGACGGAC GTGACTAAGCGAC	AGATCTCGAAGGC GCATTGGCCGTAG G	72°C Phusion 62°C ReadyMix
415_Tc_5 415_Tc_3	AGATCTGGCTCTG CTGTAGTGAGTGG	GGTACCCCTATCG TTTCCACGATCAG CG	70°C Phusion 59°C ReadyMix
end_par TnMod_ori T_3	GGCTCGAGAAGTA AGCGAGGTTGGAT GA	GGCTCGAGGCCGC CTTTTCCTCAATCG C	72°C Phusion 61°C ReadyMix
TnMod_ori T_5 TnMod_ori T_3	CGCATTAAGCATC GTCTCTCGCCTGTC CCC	GGCTCGAGGCCGC CTTTTCCTCAATCG C	72°C Phusion 65°C ReadyMix

5Z_oriR_lacZ_5 5Z_oriR_lacZ_32	CCGGTACCGCGCGTAATCTGCTGCTTGC	GAGAGACGATCGCTTACAATTTCCATTCGCC	72°C Phusion 60°C ReadyMix
RepA-F RepA-R2	GGAGATCTGGTTGCATCAGTGGCCATAGG	TCAACGTGAACGGCTCGGAG	67°C Phusion 58°C ReadyMix
RepA-F RepA-R3	GGAGATCTGGTTGCATCAGTGGCCATAGG	CACCTTCATCACCAGCAACC	64°C Phusion 54°C ReadyMix
Fdx3-f Fdx3-mr	AAGCTTGGCCCTAGCTTAAGCTACAACG	TCTAGAGACGTTCATCGTGTCCGATC	72°C Phusion 57°C ReadyMix
Fdx3-mf Fdx3-r	TCTAGAGGACACGATGAACGTCCCAAGC	GGTACCGGCGACGGTTGGTGCTAGACGC	72°C Phusion 61°C ReadyMix
parB–pSWIT02 repA-pSWIT02	GGAGATCTCCAATCTCCCCGAAGCGCGGC	GGCTCGAGGGAGATCATCGGTCAGTCGTCC	72°C Phusion 66°C ReadyMix
parA–pSWIT02 repA-pSWIT02	GGAGATCTGGTTGCATCAGTGGCCATAGG	GGCTCGAGGGAGATCATCGGTCAGTCGTCC	72°C Phusion 62°C ReadyMix
RedA2-f RedA2-mr	GAGCTCCTTGTTGAGGACACTCATGG	GGTACCGCGACCGACCAACGCGATCGAGCC	72°C Phusion 59°C ReadyMix
RedA2-mf RedA2-r	GGTACCGGCCGCAAGCTGGTCGAGGCG	GGGGATCCGCTGCTGGGTCAGCATCGATAG	72°C Phusion 67°C ReadyMix

Km3-F Km3-R	GGAGATCTGCCAC GTTGTGTCTCAAA ATC	GGCTCGAGGCTCT TAGAAAAACTCAT CGAGC	70℃ Phusion 69℃ ReadyMix
RedA1-F1 RedA1-MR	GGGAATTCGCCTT CCAGCCCGATCTT AGCTG	GGGAGCTCCGAGC TGCACGTCGACGC CATGG	72℃ Phusion 65℃ ReadyMix
RedA1-MF RedA1-R	GGGAGCTCGCGCC AAGGTCGACTGCA TCGTCG	GGGGATCCGCCAT CGGCCTCAGTCGA TCTG	72℃ Phusion 68℃ ReadyMix
Fdx1-F1 Fdx1-mr	GGGAATTCCGGAC GGTCGCGTGATGG CCGG	GGGAGCTCGGACG TGAACACGGCAGG TTGC	72℃ Phusion 68℃ ReadyMix
Fdx1-M Fdx1-R1	GGGAGCTCCTGCC GAATGGCAGGCGT GTTGC	GGGGATCCCTGCG CTGCCTTCGTGATC GGC	72℃ Phusion 68℃ ReadyMix
RedA2-R2 Km3-F	GGAGATCTGCCAC GTTGTGTCTCAAA ATC	GGCTCGAGGCTCT TAGAAAAACTCAT CGAGC	70℃ Phusion 59℃ ReadyMix
LacZmapF RepAmapR	GCAGCCTGAATGG CGAATGG	GAATGAACAGGTC GAACTCG	61℃ Phusion 50℃ ReadyMix
BlankmapF RepAmapR	CAAATGGCTGGAG ACAGTGG	GAATGAACAGGTC GAACTCG	61℃ Phusion 50℃ ReadyMix

CHAPTER 3: GENETIC CHARACTERIZATION

OF THE *SPHINGOMONAS WITTICHII* RW1

ANGULAR DIOXYGENASE SYSTEM

3.1 BACKGROUND

Oxygenases are a large class of enzymes including both monooxygenases and dioxygenases which play a key role in detoxifying organic pollutants in the environment. In general, dioxygenases have been classified into two common classes: ring-hydroxylating dioxygenases and ring-cleaving dioxygenases. The ring-hydroxylating dioxygenases are multi-component Rieske nonheme iron dioxygenase enzymes catalyzing the initial oxidation step of a wide range of natural and anthropogenic aromatic compounds and their substituted derivatives (Ashikawa et al. 2006; Barry and Challis 2013; Wammer and Peters 2006; Sauber et al. 1977).

The dihydroxylation reaction catalyzed by oxidative enzymes requires the presence of electron transfer carriers in order to shuttle the electrons from NADH to the terminal oxygenase leading to the incorporation of two oxygen atoms at two carbon molecules into the substrate (Seeger, Camara, and Hofer 2001; Bunz and Schmidt 1997; Barry and Challis 2013). However, monooxygenases enzymes consume only one oxygen atom of the diatomic oxygen molecule for the oxidation of a substrate while reducing the other oxygen atom into water. The dioxygenation reaction is a primary reaction in the degradation of many polycyclic aromatic compounds by aerobic microorganisms including such compounds as dibenzo-*p*-dioxin, dibenzofuran, naphthalene, toluene, carbazol, and fluorene (Wang et al. 2014; Barry and Challis 2013; Peng et al. 2013; Ashikawa et al. 2006).

In the dibenzo-*p*-dioxin degradation pathway, the dioxygenase system in association with the electron transport components breaks down and transforms the planar structure of the dibenzo-*p*-dioxin toxicant into harmless intermediates (Takagi et al. 2005; Kasuga et al. 2013; Hartmann et al. 2012). Therefore, the dioxygenase enzyme plays a key role in the detoxification and remediation of toxic organic pollutants from the environment (Takagi et al. 2005).

Bacterial strains have evolved two important modes of dioxygenation, lateral dioxygenation and angular dioxygenation (Nojiri, Habe, and Omori 2001). The dioxygenation reaction in the xenobiotic degraders such as *Pseudomonas* species is mostly attacking at a lateral position in the aromatic ring. However, sphingomonad species have been shown to have a mode of angular dioxygenation consisting of multiple protein components functioning together to initiate the dihydroxylation reaction (Armengaud, Happe, and Timmis 1998; Iida et al. 2002; Aly et al. 2008). More recently, several angular dioxygenases have been isolated and studied such as carbazole dioxygenase from *Sphingomonas* sp. strain CB3 and *Pseudomonas resinovorans* CA10, 3-phenoxybenzoate-1,6-dioxygenase from *Pseudomonas pseudoalcaligenes* POB310, dibenzofuran-4,4a-dioxygenase from *Terrabacter* sp. strains YK3 and DBF63, and dibenzofuran-4,4a-dioxygenase from *Sphingomonas wittichii* RW1 (Armengaud, Happe, and Timmis 1998; Nojiri, Habe, and Omori 2001; Kasuga et al. 2013; Habe et al. 2001; Takagi et al. 2005). These types of dioxygenases are commonly known as Rieske non-heme iron oxygenases consisting of a catalytic iron-sulfur protein with a Rieske-type [2Fe-2S] center (the terminal oxygenase component) and electron transfer proteins (Wang et al. 2014; Aly et al. 2008; Ashikawa et al. 2006).

The catalytic attack of the aromatic moiety by the ring-hydroxylating dioxygenase is not yet fully understood. However, previous studies have described a proposed reaction of the aromatic ring hydroxylation. First, the aromatic substrate binds to the mononuclear iron of the oxygenase component. Then, ferric iron is formed by binding one oxygen atom of the diatomic oxygen to the mononuclear iron and donating an electron from the Rieske center to the specified mononuclear iron. The ferredoxin component donates an electron to the mononuclear iron which leads to the formation of the ferrous iron and releasing the dihydrodiol product from the cleavage of the Fe—O bond. Then the ferredoxin donates a second electron to the Riske center forming a ferrous iron where the hydroxylase is at its initial step and ready to repeat the step again.

Sphingomonas wittichii RW1 is well known as a dibenzo-*p*-dioxin mineralizing bacteria that was isolated for the first time from the Elbe River (Wittich et al. 1992). Genes involved in the dibenzo-*p*-dioxin degradative pathway can be divided into two groups, the upper pathway genes, and the lower pathway genes. The initial dioxygenase genes, the meta-cleavage genes, and the hydrolases are responsible for encoding the upper pathway. Most of these genes are well studied and characterized. On the other hand, the genes that are involved in the lower pathway part include all the genes responsible for converting the upper pathway intermediates into Krebs cycle intermediates.

In *S. wittichii* RW1, the biodegradation of dibenzo-*p*-dioxin and dioxin-like compounds is initiated by an unusual enzyme system called dibenzofuran-4,4a-dioxygenase (Bunz and Cook 1993; Happe et al. 1993). This type of dioxygenase requires a unique type of ferredoxins called a putidaredoxin-type [2Fe-2S] ferredoxin

rather than a Rieske-type [2Fe-2S] ferredoxin which is known to provide electrons to the common dioxygenase such as class IIB and class III dioxygenase (Armengaud and Timmis 1998, 1997). Because of the highly stable structure and the toxic nature of dibenzo-*p*-dioxin, this first step is considered as the most important step in the biodegradative pathway.
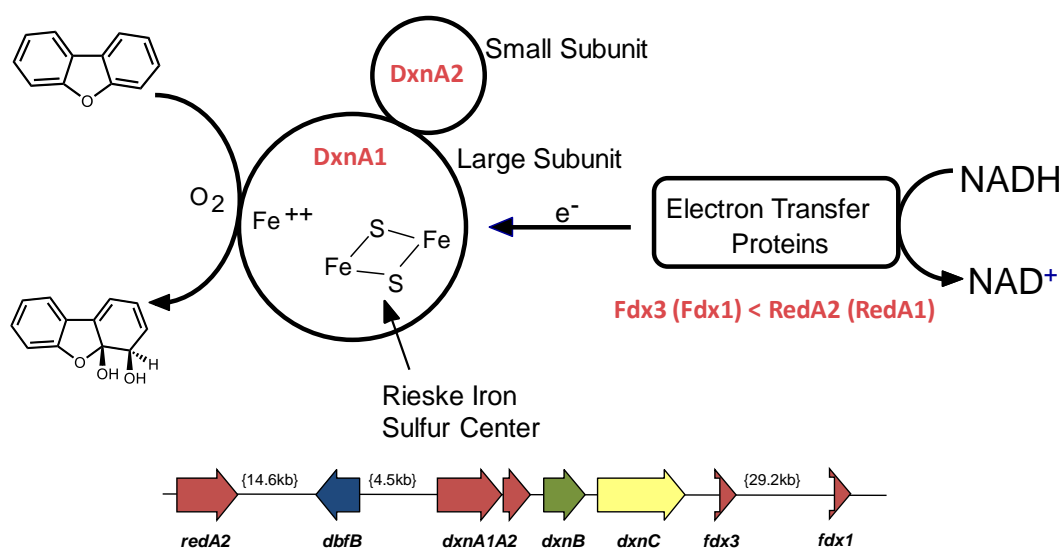
Figure 5. A schematic of the angular dioxygenation reaction.

The dioxin dioxygenase was first purified and biochemically analyzed by Bunz and Cook in 1993 as a key enzyme responsible for the initial step of dioxin metabolism (Bunz and Cook 1993). The dioxin dioxygenase is a constitutive enzyme system that functions through a complex comprised of four important degradative genes, the *dxnA1A2* encoded oxygenase, a reductase, and a ferredoxin. This reaction requires the transfer of electrons from NADH to the electron supply system: the reductase and the ferredoxin and finally to the oxygenase that adds two hydroxyl groups to the aromatic ring when it is activated. This step results in the formation of an unstable compound called a *cis*-dihydrodiol that coverts to a trihydroxy intermediate. The aromatic ring is subsequently cleaved by a ring cleavage enzyme that breaks one of the aromatic rings to yield 8-OH 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPD). A hydrolase then catalyzes a reaction to form catechol that is further channeled and degraded into TCA cycle intermediates.

Based on the electron transfer components properties, Batie et al. have classified the multicomponent dioxygenases into three major classes, class I, class II and class III. Each of these three classes is subdivided into two classes A and B. Class IA and class IV varies based on the redox center where class IA contains flavin mononucleotide FMN whereas class IB contains flavin adenine dinucleotide. Similarly, class IIA is known to have putidaredoxin ferredoxin instead of the Rieske center found in class IIB (Armengaud and Timmis 1998). Based on the proposed classification system of Batie et al., the dioxin dioxygenase was classified to belong to the class IA ring-hydroxylating dioxygenases due to the biochemical properties of the ferredoxin gene where the prosthetic group 2Fe-2S is organized with four cysteine residues.

The two flavoprotein reductase components RedA1 and RedA1 and one ferredoxin named fdx1 were first cloned and studied from dibenzofuran-grown RW1 by Bunz and Cook in 1993 (Bunz and Cook 1993) and further characterized by Armenguad and Timmis in 1998 (Armengaud, Happe, and Timmis 1998; Armengaud and Timmis 1997). The structure of the ferredoxin component associated with the angular dioxygenase displays a unique type of electron transfer carriers called a putidaredoxin-type [2Fe-2S] ferredoxin since the [2Fe-2S] cluster is ligated to four cysteine rather than two histidine and two cysteine residues found in Rieske type ferredoxins (Armengaud and Timmis 1997, 1998). The genes for the two ferredoxins were found in two loci in RW1. The *fdx3* gene is located in an operon with the *dxnA1A2* oxygenase component genes while the *fdx1* gene is located elsewhere on pSWIT02 in RW1. The Fdx1 was the first electron transfer component associated with dibenzo-*p*-dioxin dioxygenase to be isolated and identified through hyperexpression experiment. The purified Fdx1 was found to function as an electron carrier to the dibenzo-*p*-dioxin dioxygenase. A second ferredoxin component named Fdx3 functioned as an electron donor with the dioxygenase and was isolated and purified by Armenguad et al. in 2000 based on its properties that match the previously isolated Fdx1 component (Armengaud, Gaillard, and Timmis 2000). This ferredoxin was also biochemically characterized and found to also contain a putidaredoxin-type 2Fe-2S cluster rather than the Rieske type 2Fe-2S cluster (Armengaud, Gaillard, and Timmis 2000). From the N-terminal sequence, they found that this ferredoxin exhibits moderate homology (35-45%) with putidaredoxin-type ferredoxin from *Pseudomonas putida* and ferredoxin from *Rhodococcus* sp. strain (Armengaud and Timmis 1997). The flavoprotein reductase RedA2 was found to share a moderate

homology (about 40%) with class-I cytochrome P450 reductase (Armengaud and Timmis 1998).
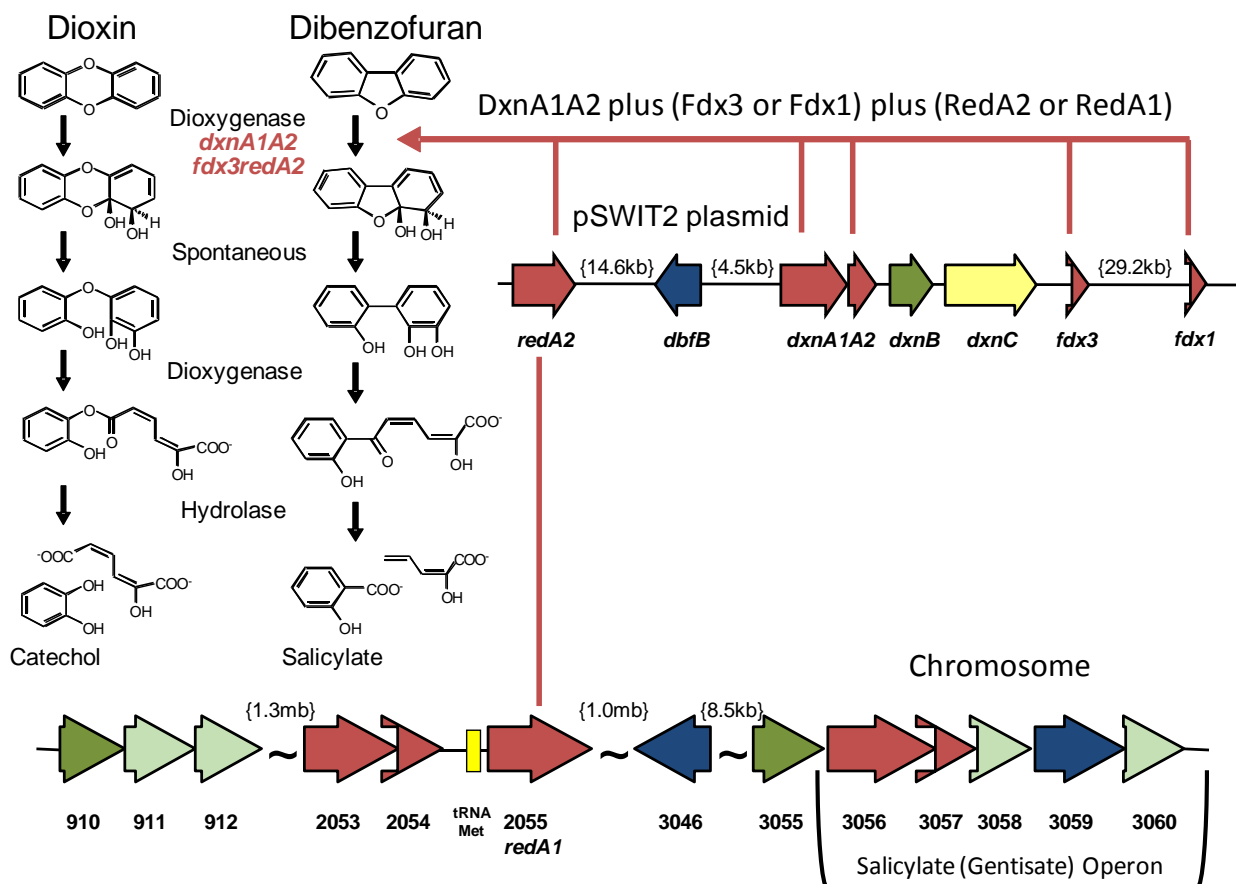
Figure 6. Metabolic Map of RW1 DBF and DXN Degradation

3.2. GENE KNOCKOUT

Gene knockout is defined as the inactivation of a targeted gene function by introducing a mutation in that gene using different DNA recombining techniques. Over recent years, gene knockout technology has been widely used in a variety of organisms including both prokaryotic and eukaryotic organisms (128). Generating a knockout mutant was first described in *E coli* by Hamilton et al. in 1989 (129) and since then a numerous number of knockout mutants have been reported in a wide variety of bacterial species (130). In bacteria, generating a knockout mutant has been considered as one of the most efficient ways and a powerful tool to elucidate gene function by deactivating the targeted gene. It helps better understand the molecular mechanism and the function of unknown genes.

A gene knockout can be obtained using different approaches of genetic modification such as homologous recombination strategy, NHEJ technique or a CRISPR-CAS9 method. In the homologous recombination-based-method, the knockout is accomplished by designing a plasmid construct that carries a DNA sequence of the gene of interest flanked by a homologous sequence. The sequence of the gene is disrupted by inserting an antibiotic marker into the gene of interest. When the final construct is moved into the recipient strain the homologous ends will recombine with the original sequence in the wild type strain by homologous recombination resulting in a knockout mutant strain with nonfunctional gene. In our study, generating single and double knockout mutants was efficiently used to detect essential genes involved in the catabolic pathways and reveals that multicopy genes of the electron transfer carriers participate in the hydroxylation reaction of dibenzo-*p*-dioxin molecule.

3.3 GENERATING A KNOCKOUT MUTANT OF THE REDUCTASE AND FERREDOXIN.

Abstract

Sphingomonas wittichii RW1 is of a great interest for its diverse metabolic activities. *S. wittichii* RW1 uses a unique angular dioxygenase system to break down the recalcitrant carbon structure of dioxin. Previous studies showed that the RW1 dioxygenase possibly functions with multiple ferredoxins and reductases. However, how many genes are actually involved in the electron transfer remains to be elucidated. Here we report the characterization of the essential electron carrier proteins, a ferredoxin and a reductase, implicated in dioxin metabolism by generating single and/or double knockout mutants of each electron transfer component. The single and double knockout mutants were examined on minimal medium supplemented with dibenzo-*p*-dioxin or dibenzofuran for growth ability. Screening tests revealed that either RedA1 or RedA2 and Fdx1 or Fdx3 are able to serve as an electron donor and to oxidize NADH in the first step of the dioxin degradation pathway. The single knockout mutant of each of the electron transfer components showed no effect when *S. wittichii* RW1 was grown on dibenzo-*p*-dioxin or dibenzofuran while the double knockout mutant of the same component abolished growth on both of the substrates, confirming that two electron carrier proteins, Fdx1 and Fdx3, RedA1 and RedA2 participate in transferring electrons to the initial dioxygenase. This proves that the two ferredoxins and the two reductases are reciprocal in their function. Our work provides in vivo physiological proof of the role of the two

reductases and two ferredoxins in dioxin metabolism thus complementing the previous in vitro protein purification experiments.

3.4. RESULTS

Unlike the lateral dioxygenation which several bacterial species rely on for the utilization of the aromatic compounds, only a few bacterial strains have the ability to use angular dioxygenation in aromatic compound degradation. Among the best studied dioxygenases systems isolated so far is the dioxin dioxygenase of the bacterium *Sphingomonas wittichii* strain RW1. The dioxin dioxygenase catalyzes the initial attack of both dibenzo-*p*-dioxin and dibenzofuran compounds. The genes that are involved in this step of dioxin metabolism include *dxnA1A2* encoding the two angular oxygenase subunits, *redA1* and *redA2* encoding two different reductases, and *fdx1* and *fdx2* encoding two different ferredoxins. This dioxygenase system is characterized by its unique mode of dioxygenation that attacks at an angular position (4,4a) on the ether bridge of dibenzo-*p*-dioxin. The dioxygenation reaction is achieved through the inserting two hydroxyl groups into the dioxin molecule by an angular dioxygenase. This specific reaction requires the oxidation of NADH via electron transfer carriers that involve a reductase and ferredoxin. The electrons therefore shuttle down to the terminal oxygenase that attacks the dioxin molecule by adding two hydroxyl groups, with one of them at the angular position on the aromatic rings. Several studies have been reported on the enzymatic steps of the dibenzo-*p*-dioxin degradation pathway. The molecular and biochemical studies revealed that this microorganism contains multiple copies of the putative genes that are involved in the dibenzo-*p*-dioxin metabolism pathway. For example, two reductases and two ferredoxins have been identified to serve as electron carriers in the dibenzo-*p*-dioxin

degradation pathway. The first ferredoxin named Fdx1 has been purified and studied by Armengaud and Timmis (1997). From the N-terminal sequence of the purified protein, they recognized this protein as a putidaredoxin-type [2Fe-2S] ferredoxin that shares significant similarity (40%) with other known ferredoxins of different species of bacteria.

In order to study the redundancy of these important genes involved in the degradative pathways of dibenzo-*p*-dioxin and dibenzofuran, generation of knockout mutants of each of these genes was attempted in order to investigate the function of the encoded proteins. To achieve this we isolated the targeted gene from the whole genomic DNA of *S. wittichii* RW1 and cloned it into an unstable cloning vector. The resulting clone was then moved into the recipient RW1 strain and the mutant strain was obtained via homologous recombination of the inactivated gene with original wild type gene. The mutant strain was first screened on LB kanamycin plates and LB tetracycline plates. The colonies that grow on kanamycin but not tetracycline are the desired mutant strains. The knockout mutant was then confirmed by PCR. Then the knockout mutants were tested for growth ability on dibenzo-*p*-dioxin and dibenzofuran. The screening test showed that the oxygenase component knockout RW1ΔdxnA1A2 did not grow on either dibenzo-*p*-dioxin or dibenzofuran. This indicates that the RW1 strain contains only one functional oxygenase component to initiate dibenzo-*p*-dioxin and dibenzofuran metabolism. On the other hand, the single knockout mutants of each of the ferredoxin genes (RW1Δfdx1 and RW1Δfdx3) and the reductase genes (RW1ΔredA1 and RW1ΔredA2) were still able to grow on both dibenzo-*p*-dioxin and dibenzofuran as the sole carbon and energy source. However, the double knockout mutants RW1Δfdx1Δfdx3 and RW1ΔredA1ΔredA2 lost the ability to grow on dibenzo-*p*-dioxin. and dibenzofuran. This proves that the two

reductases and the two ferredoxins can replace the function of each other and there are only two ferredoxins and two reductases in RW1 that can serve as electron carriers. The mutant strains were then complemented by the analogous cloned genes which restored the growth ability on the two aromatic substrates showing that no other genes were affected by the knockout mutataions.

To further confirm these results and to examine the phenotype of the generated mutant strain, a growth curve has been performed for each of the knockout mutants to test the growth rate of the mutant strain in comparison with the wild type strain. The mutant strains were subcultured into minimal medium supplemented with 3 mM dibenzo-*p*-dioxin or dibenzofuran. The growth curves show that the single knockout mutants grow as fast as the wild type strain. However, the double knockout mutants did not grow at all on either substrate. This confirms the plate screening test and indicates that the RW1 harbors two genes encoding isofunctional ferredoxins and two genes encoding isofunctional reductases.

3.5. DISCUSSION

The dioxin degradation pathway in *Sphingomonas wittichii* RW1 occurs via a dihydroxylation reaction of the dibenzo-*p*-dioxin molecule by catalysis of a typical dioxygenase system called an angular dioxygenase functioning with electron transfer components, a reductase and a ferredoxin. In this important first metabolic step, the dibenzo-*p*-dioxin molecule is hydroxylated at two neighboring carbon atoms forming an unstable hemiacetal compound. This is followed by a spontaneous reaction which forms chemically stable compounds including, 2,2',3-trihydroxybiphenyl for the dibenzofuran pathway and 2,2',3-trihydroxybiphenyl ether for the dibenzo-*p*-dioxin pathway. This is

followed by ring cleavage of one aromatic ring to produce a compound called HOPDA.

The latter compound then undergoes a hydrolysis reaction producing catechol from

dibenzo-*p*-dioxin or salicylate from dibenzofuran. Based on previous biochemical work

we know that there are multiple potential electron transfer components that are actively

functioning to provide electrons to the terminal oxygenase. The first identification of

these proteins was performed by Bunz and Cook in 1993 who demonstrated the presence

of a putative dioxygenase enzyme and a reductase termed RedA1 and a ferredoxin

designated Fdx1 (Bunz and Cook 1993). A few years later, an enzymatic analysis was

done by Armenguad et al. 2000 on the characterization of the important catabolic genes

involved in the first step of dioxin metabolism (Armengaud, Gaillard, and Timmis 2000).

They have successfully demonstrated the genetic and biochemical analysis of the

previously isolated electron transfer components. The multicomponent dioxin

dioxygenase system was found to be carried on one distinct locus of the megaplasmid

pSWIT02. However, the reductase RedA2 has been found to be located on a neighboring

genomic segment on the megaplasmid and transcribed opposite to the genes for the

dioxygenase system. Additionally, they found a second copy of the ferredoxin named

fdx3 that can be used by the dioxygenase as an efficient electron transfer carrier

(Armengaud, Gaillard, and Timmis 2000). Other studies based on the proteomic analysis

suggested that a third reductase identified as Swit_0104 may also participate in the

electron transfer to terminal oxygenase. In this work, therefore, we sought to determine

the exact copies of these important genes in *Sphingomonas wittichii* RW1. We

successfully obtained a single knockout mutant of each of the reductase genes *redA1* and

redA2. We also obtained a single knockout mutant of each of the ferredoxin genes *fdx1*

and *fdx3*. Our data showed that each of the single knockout mutants was able to provide electrons to the dioxin dioxygenase and thereby allowed the growth of RW1 on both dibenzo-*p*-dioxin and dibenzofuran. However, by generating double knockout mutants of the two reductases and testing the mutant strains for their growth ability we noticed that the mutant has lost the ability to grow on both dibenzo-*p*-dioxin and dibenzofuran. Similarly, a double knockout mutant of the two ferredoxin genes could not use dibenzo-*p*-dioxin or dibenzofuran as a sole carbon and energy source. These data provide proof that *S. wittichii* RW1 contains two reductases and two ferredoxins that function as putative electron transfer components in the dioxygenolytic attack of the dioxin compound.
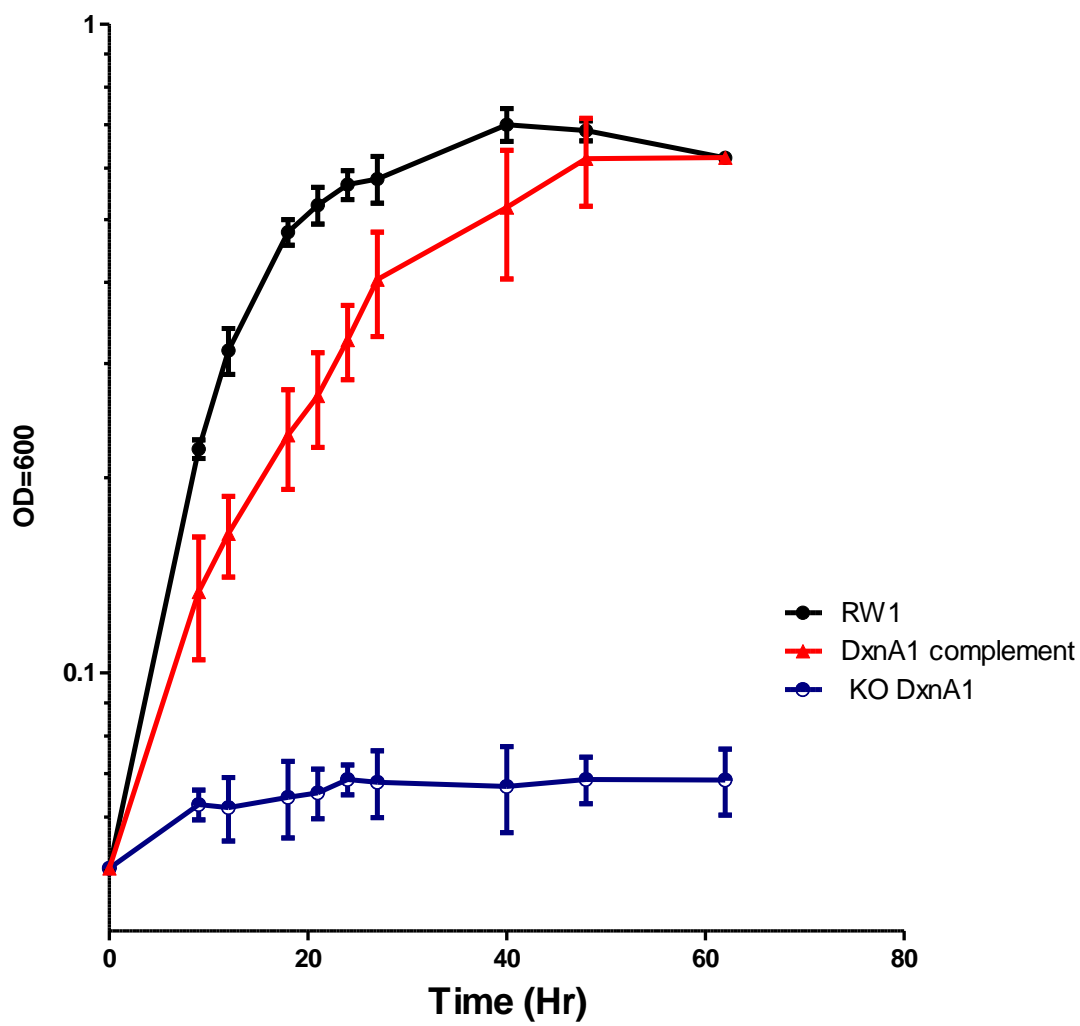
Figure 7. Growth of DxnA1 knockout mutants on DD

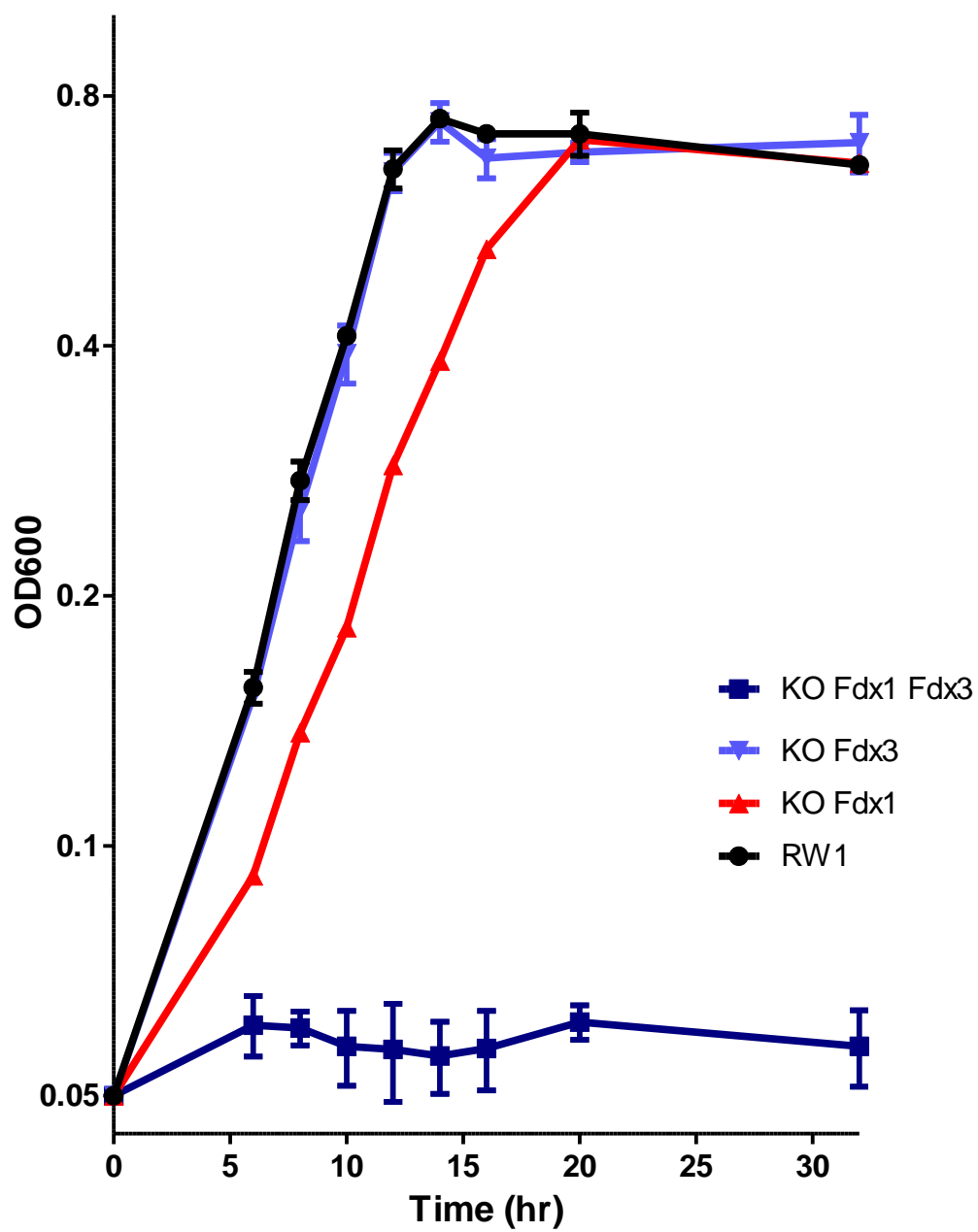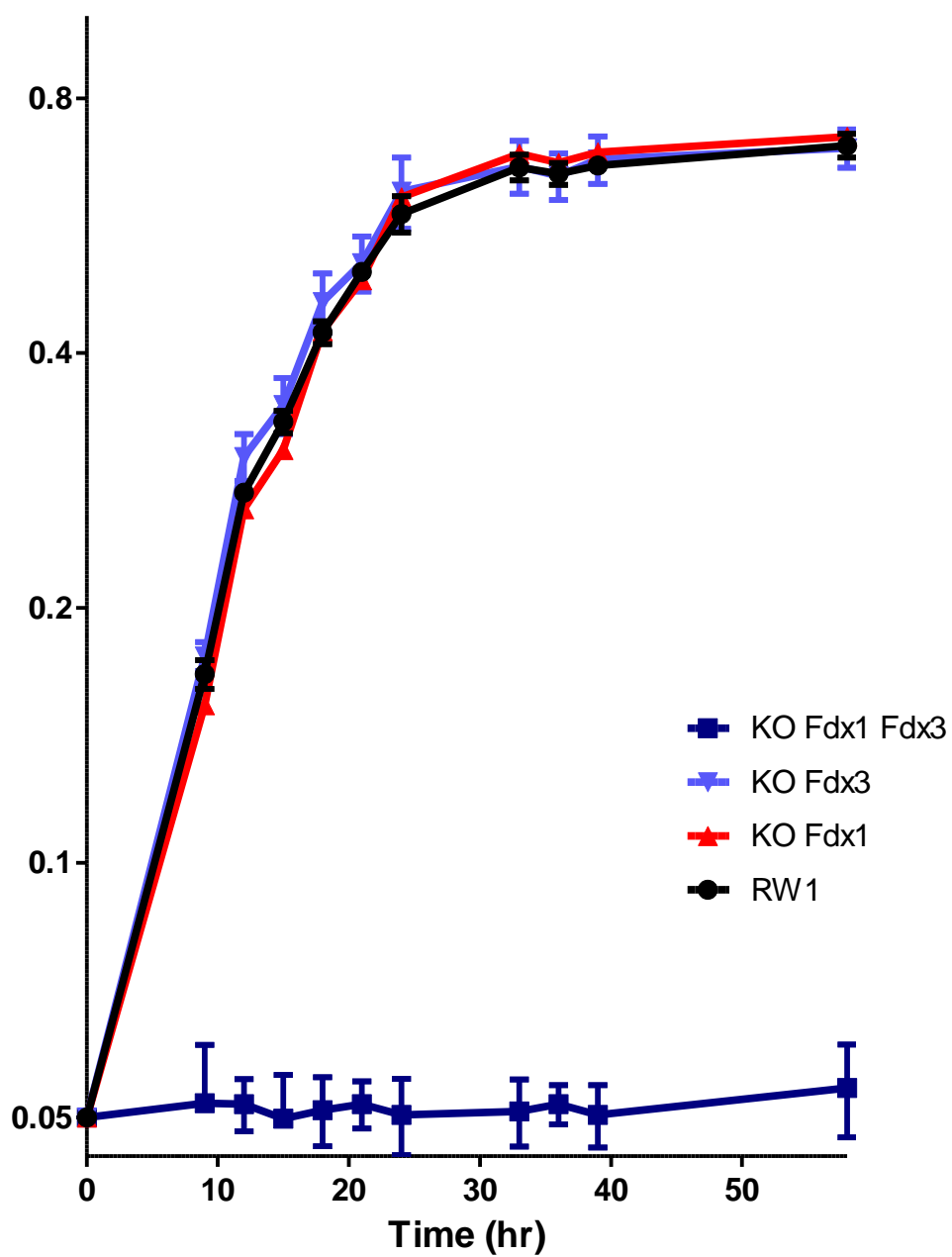**Figure 8. Growth of Fdx1 and Fdx3 knockout mutants on DBF**

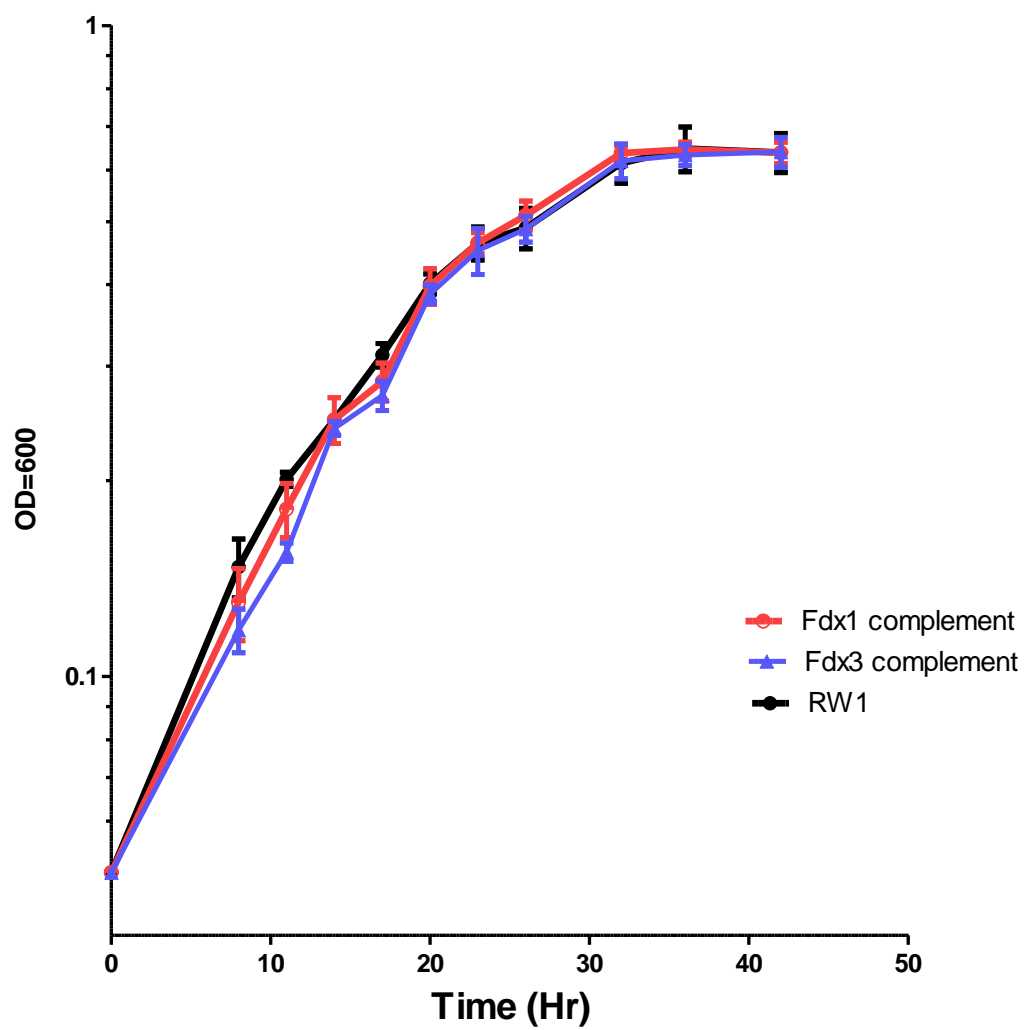**Figure 9. Growth of Fdx1 and Fdx3 knockout mutants on DD**

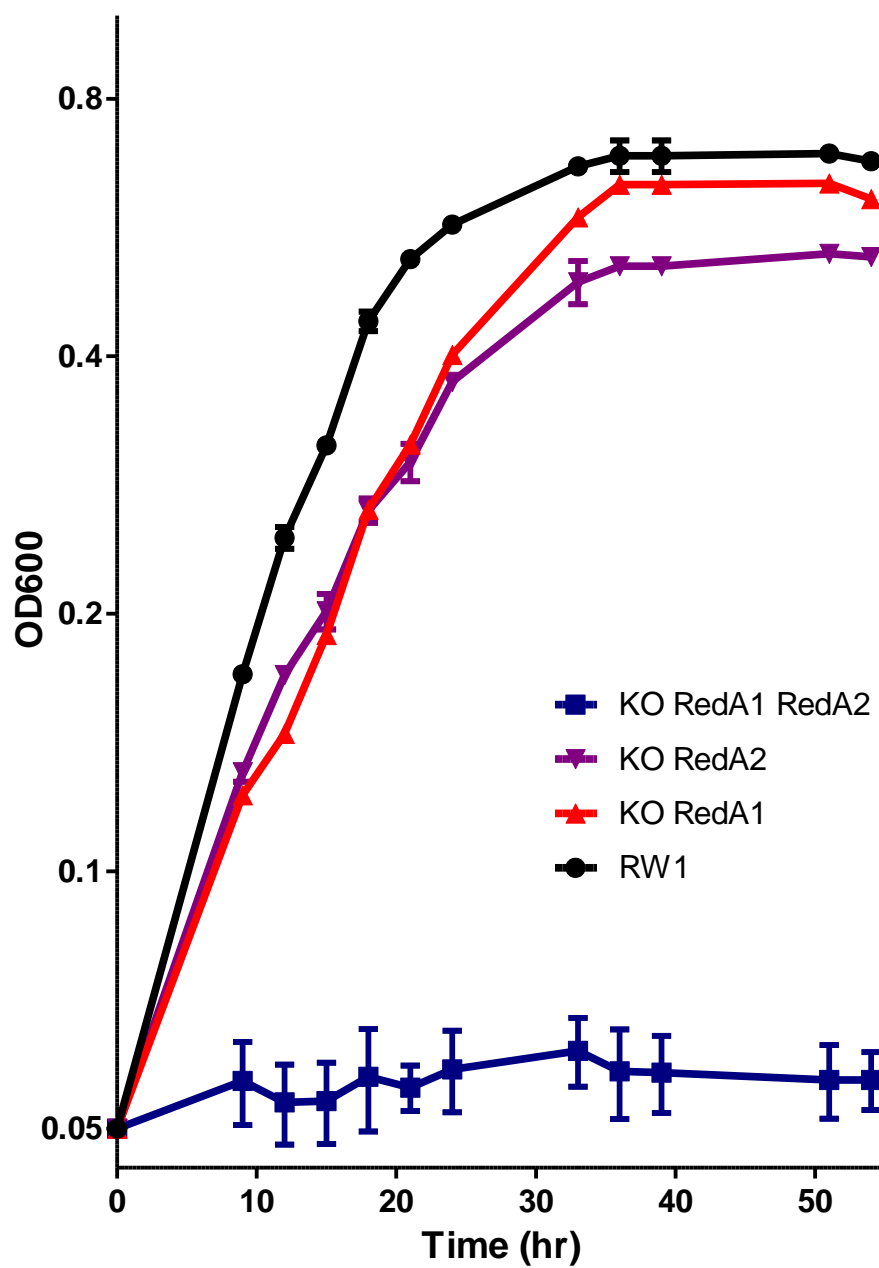**Figure 10. Growth of Fdx1 and Fdx3 complements on DD**

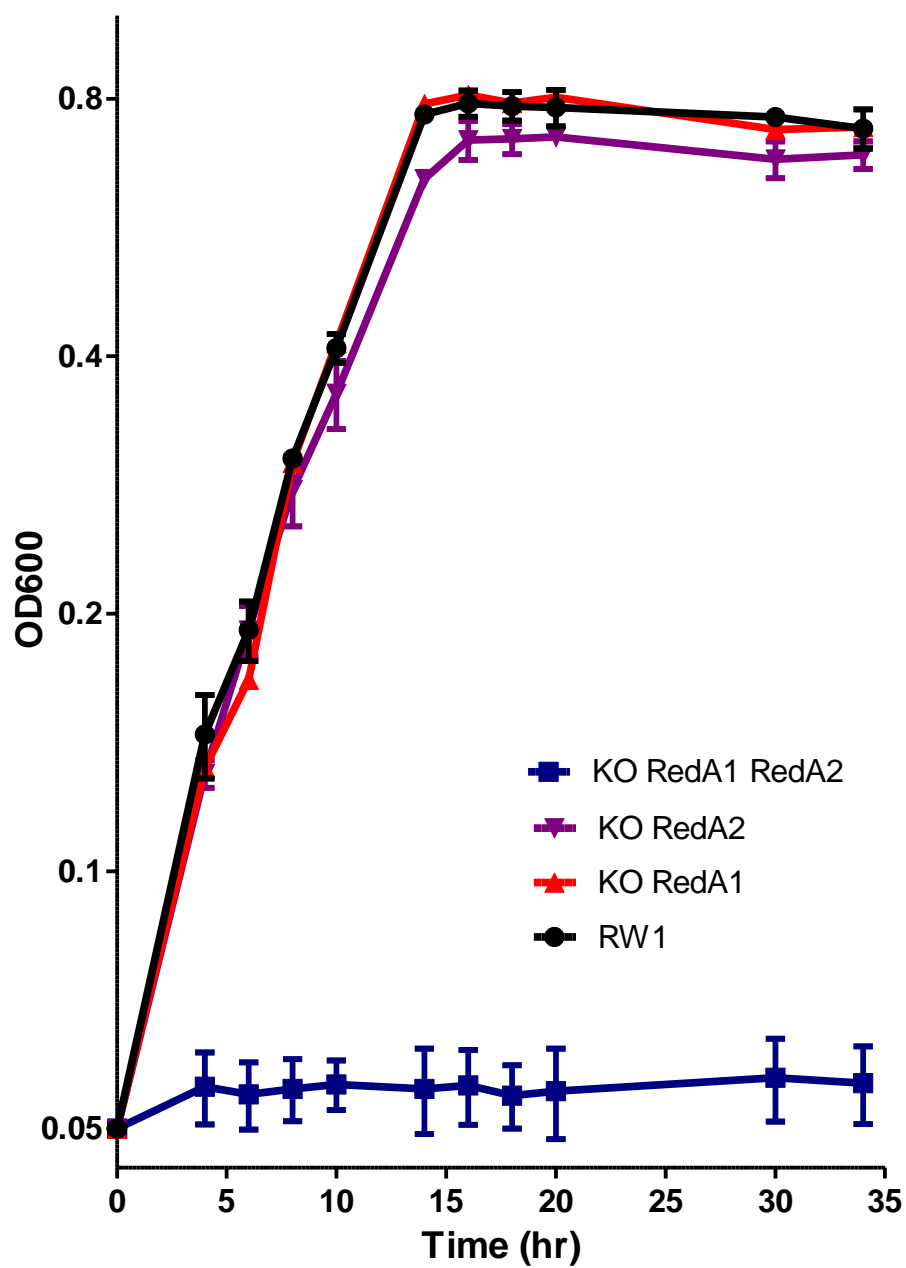**Figure 11. Growth of RedA1 and RedA2 knockout mutants on DD**

Figure 12. Growth of RedA1 and RedA2 knockout mutants on DBF
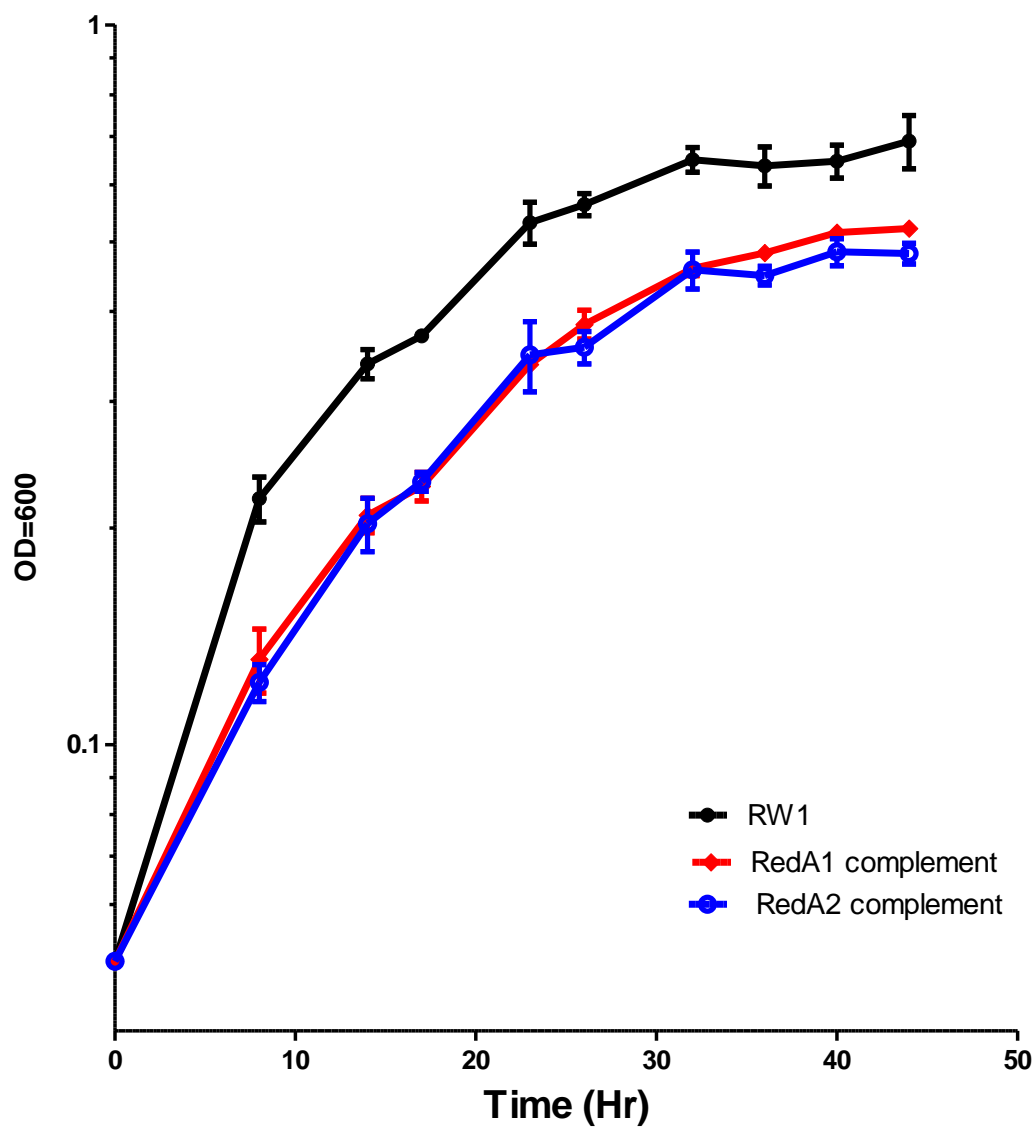
Figure 13. Growth of RedaA1 and RedA2 complements on DD
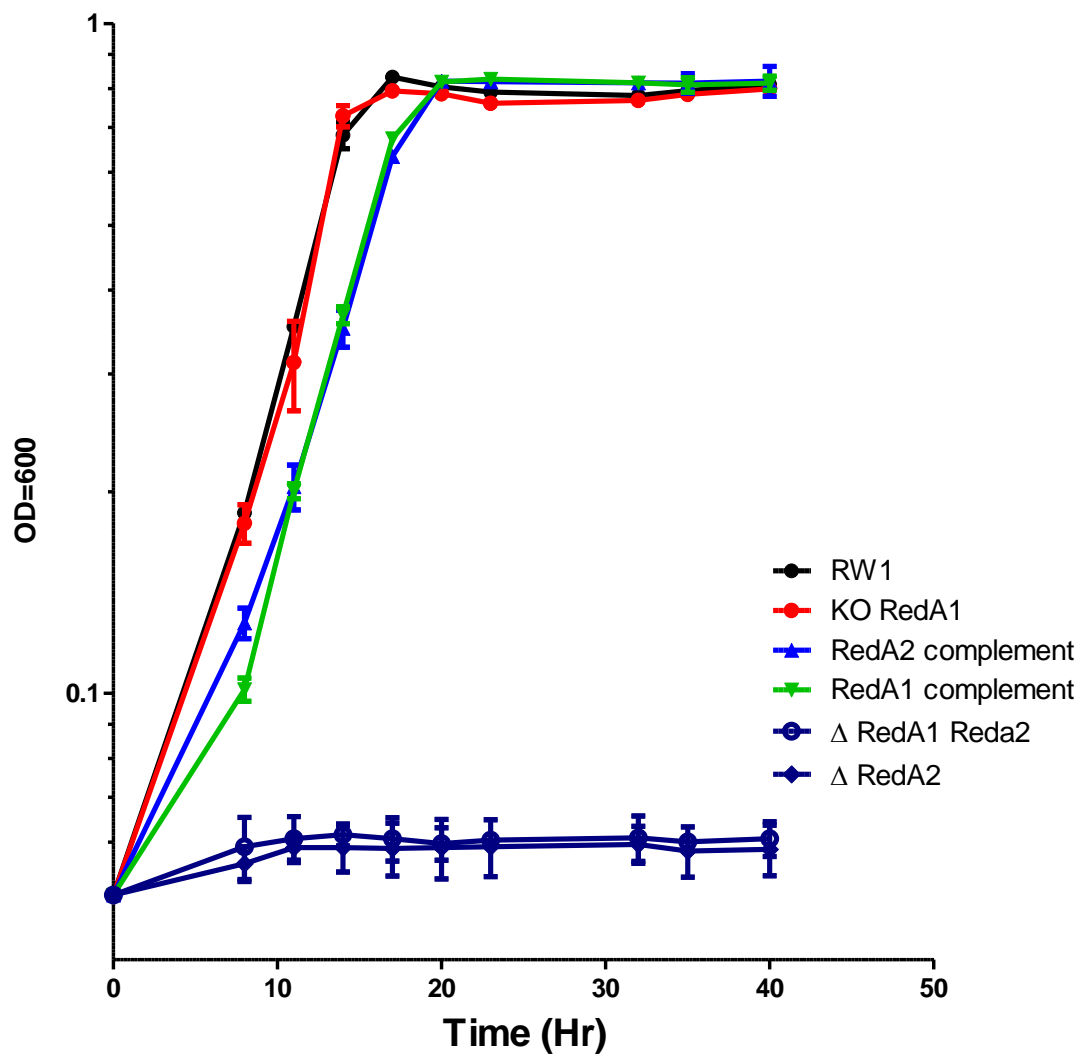
Figure 14. Growth of RW1 on 5 mM salicylate

To test whether the two reductases RedA1 and RedA2 are also involved in the electron transport for dioxygenases other than the angular dioxygenase DxnA1A2, the single knockout mutants RW1ΔredA1 and RW1ΔredA2 were grown on MSB agar plates supplemented with 5 mM salicylate and incubated at 30°C for four days. Interestingly, the RW1ΔredA1 knockout mutant showed normal growth on salicylate medium but the RW1ΔredA2 knockout mutant was no longer able to grow on salicylate. This proves that the second reductase RedA2 supplies electrons to the dibenzo-*p*-dioxin dioxygenase as well as to the salicylate oxygenase. To better clarify the function of the reductase enzyme, a complementation test was performed of the RedA2 mutant strain. The wild type *redA2* reductase gene was cloned into the pRK415 broad host vector and the clone was then moved into the RW1ΔredA2 knockout mutant by triparental mating. The complemented strain restored the ability to grow on MSB medium with salicylate. A growth curve was performed to measure the growth rate of the mutant strain and the complemented strain. The double knockout mutant RW1ΔredA1ΔredA2 was complemented with either cloned *redA1* or *redA2*. Interestingly, when we complemented the double knockout mutant with either of the two reductases the cloned genes restored the growth on salicylate in both cases. Our hypothesis is that the *redA1* gene is expressed constitutively on the plasmid pRK415 and became functional while the wild type *redA1* gene is not expressed when the wild type strain grows on salicylate. These findings prove that the salicylate oxygenase requires a reductase as an important electron carrier in the initial dihydroxylation attack and both the dibenzo-*p*-dioxin and the salicylate oxygenase share the same reductase component for this critical step. In addition, the reductase knockout mutant was also tested on MSB with benzoate as the sole carbon and energy

source. The single knockout mutant of the RedA2 gene showed a normal growth on

MSB/benzoate plate after 3 days incubation at 30 C. However, the mutant RW1 strain

lacking both of the reductases genes was no longer able to utilize benzoate as the only

carbon source and the growth was completely abolished on this substrate. These findings

show that each of the dioxin dioxygenase, the salicylate oxygenase, and the benzoate

oxygenase compete for the same electron transport component, in particular, the

reductase component RedA1 and RedA2.
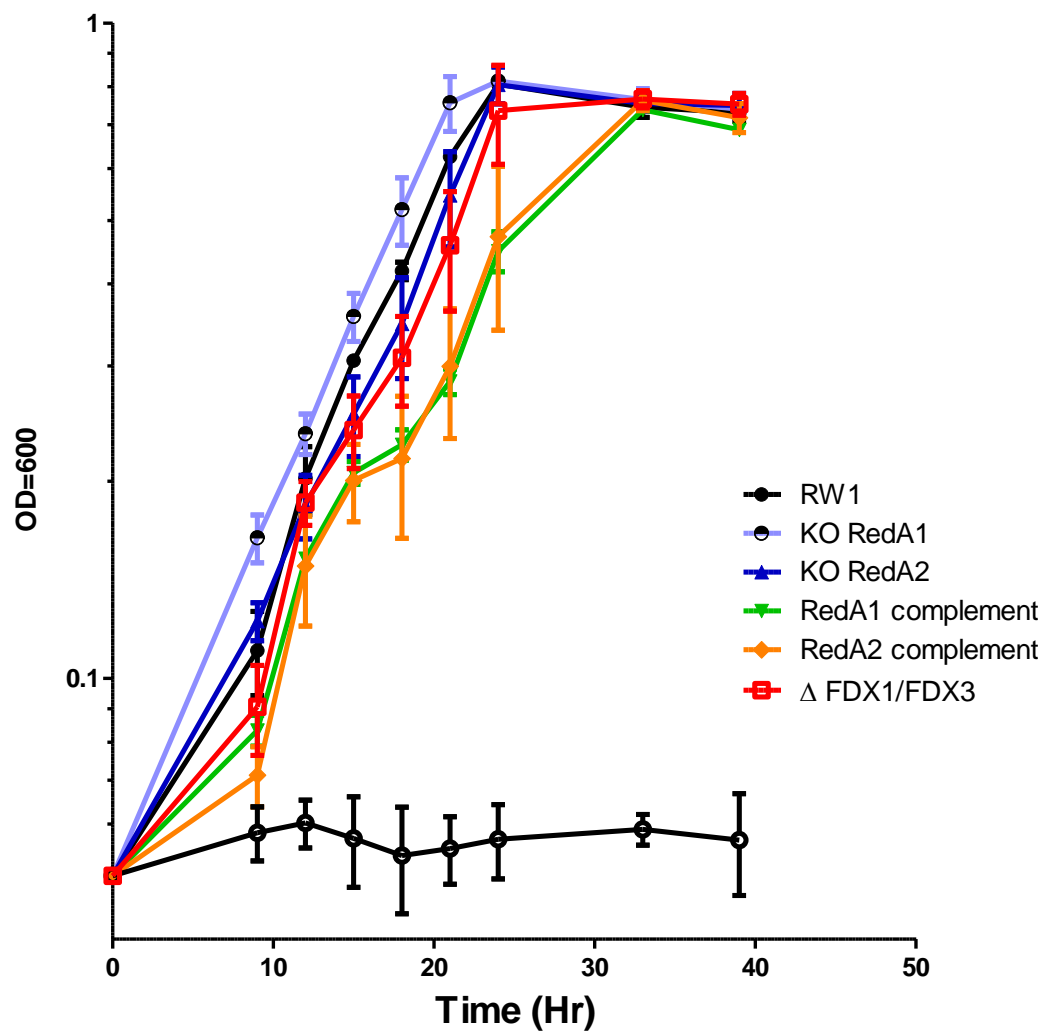
Figure 15. Growth of RW1 on 5 mM benzoate

Dioxygenase Component	Generic Name	Genome Location	Sequence SWIT#	Growth on Dibenzofuran	Growth on Dioxin
Oxygenase	DxnA1A2	pSWIT02	4897	-	-
Ferredoxin	Fdx3	pSWIT02	4893	+	+
	Fdx1	pSWIT02	5088	+	+
	Double Knockout			-	-
Reductase	RedA2	pSWIT02	4820	+	+
	RedA1	Chromosome	2055	+	+
	Double Knockout			-	-

Table 2: Summary of the RW1 DBF/DD Dioxygenase Knockout

CHAPTER 4: GENETIC MANIPULATION OF

SPHINGOMONAS WITTICHII RW1

4.1. ABSTRACT

Sphingomonads are well known for their potential in the bioremediation field and their unusual capability to metabolize a wide range of monocyclic and polycyclic aromatic compounds including benzene, toluene, xylenes, phenols, biphenyl, naphthalene, phenanthrene, carbazole, dibenzofurans, and dibenzo-*p*-dioxins. *Sphingobium yanoikuyae* strain B1 is one of the most interesting *Sphingomonas* strains because of its capability to degrade a broad variety of monocyclic and polycyclic aromatic hydrocarbons such as biphenyl, naphthalene, phenanthrene, toluene, and *m*- and *p*-xylene as the sole source of carbon and energy. The complete sequence of the 32 kb *Sphingomonas yanoikuyae* B1 cryptic plasmid pKG2 revealed an origin of replication sequence (*oriR*) and two essential genes encoding for replication and partitioning components of the plasmid, named *repA* and *parA* respectively. We hypothesized that the *repA* gene along with the origin of replication (*oriR*) is the minimum required for replication of pKG2. We also hypothesized that *parA* is required for stability of the plasmid and proper partitioning during cell division. We designed and constructed two *Sphingomonas* shuttle vectors, one containing *repA* and *oriR* from pKG2 and the other containing *repA*, *oriR*, and *parA* from pkG2. Additionally, the plasmids contained a tetracycline resistance gene, an origin of conjugal transfer (*oriT*), the *E. coli* pMB1 *oriR*, and a *lacZ* gene with a multiple cloning site from pGEM5Z(f-). The newly constructed vectors were then examined for their ability to be maintained in *Sphingomonas* strains.

Upon introduction into *S. yanoikuyae* B1 with tetracycline selection both new vectors

caused the loss of plasmid pKG2 confirming the expected incompatibility due to the

identical *oriR* in both plasmids. Plasmid stability tests showed that the *repA-oriR-parA*

vector was completely stable (0% loss) over six days of subculture without antibiotic

selection. In contrast, the *repA-oriR* vector was lost at the rate of 10% for every 24 hr

subculture. Our work demonstrates that the *repA-oriR* region is sufficient for replication

and that *parA* is absolutely required for plasmid stability. These two cloning vectors in

both a stable and unstable version are useful genetic tools for the manipulation of

Sphingomonas strains.

4.2. SPHINGOMONAD MEGAPLASMIDS

A number of bacterial strains are known to harbor large circular or linear plasmids

with sizes over 100 kilobases known as large plasmids or megaplasmids (131). These

extrachromosomal DNA molecules confer significant traits for host bacteria such as

antibiotic resistance, symbiosis, virulence, nitrogen fixation or xenobiotic compound

degradation (132-134). Several degradation plasmids have been reported in different

bacterial strains, specifically those that belong to the alphaproteobacteria family include

plasmids such as pCAR3 of *Sphingomonas* sp. strain KA1 (135), pNL1 of *Sphingomonas*

aromaticivorans F199, pSymA and pSymB of *Sinorhizobium meliloti* 1021 (133), and the

pSWIT02 plasmid in *S. wittichii* RW1. These type of plasmids are commonly known as

repABC plasmids. The *repABC* operons encode three proteins known as RepA, RepB,

and RepC. While the *repA* and *repB* genes encode proteins responsible for plasmid

partitioning, *repC* encodes a protein for replication. The megaplasmids pCAR3 and

pSWIT02 contain a repeat DNA sequence known as iterons, between 14-17 bp long

which is a common characteristic of plasmids belonging to RepABC family within the RepA-group (136). In particular, large plasmids are frequently found in members of sphingomonads that play an essential role in dibenzo-*p*-dioxin and/or dibenzofuran metabolism conferring a unique characteristic of *Sphingomonas* members (131, 136, 137). Most of the sphingomonad megaplasmids encode important catabolic genes known to be involved in the degradation pathway of polyaromatic hydrocarbons. Therefore, megaplasmids contribute to the survival and adaptation of sphingomonad strains in contaminated environments with organic pollutants.

The sequences of some of these large plasmids have been completed and analyzed (138). Sequence analysis of the large plasmids showed that catabolic genes are one of the dominant sequences in the large plasmids and most of the identified ORFs are related to aromatic compound degradation. For instance, the megaplasmid pNL1 from *Sphingomonas aromaticivorans* was found to have an essential role in the biodegradation of PAHs such as naphthalene and biphenyl and pSWIT02 from *Sphingomonas wittichii* RW1 has been shown to have a crucial role in dibenzo-*p*-dioxin metabolism (136).

In recent years, intensive work has been done to detect the function of the large plasmids in several *Sphingomonas* strains particularly in the bacterium *Sphingomonas wittichii* RW1. Among these studies, protein purification experiments revealed the function of a number of the important catabolic genes that are located on the megaplasmid pSWIT02 such as the angular dioxygenase system, the meta-cleavage enzyme, and the hydrolases. In order to prove the important function of the megaplasmid, we sought to design a new shuttle vector that enables the loss of pSWIT02 based on the incompatibility of the introduced new vector and the original RW1 megaplasmid. A

previously designed vector termed pSEZ_B1RP was utilized to design a useful

Sphingomonas vector carrying the same origin of replication of the megaplasmid

pSWIT02. The new *Sphingomonas* vector named pSEZ_RW1RP was successfully

utilized to determine the function of the important pathway genes for dibenzo-*p*-dioxin

and dibenzofuran metabolism by eliminating the megaplasmid pSWIT02, which is

known for its important function as a degradative plasmid carrying several important

catabolic genes required for both dibenzo-*p*-dioxin and dibenzofuran metabolism.
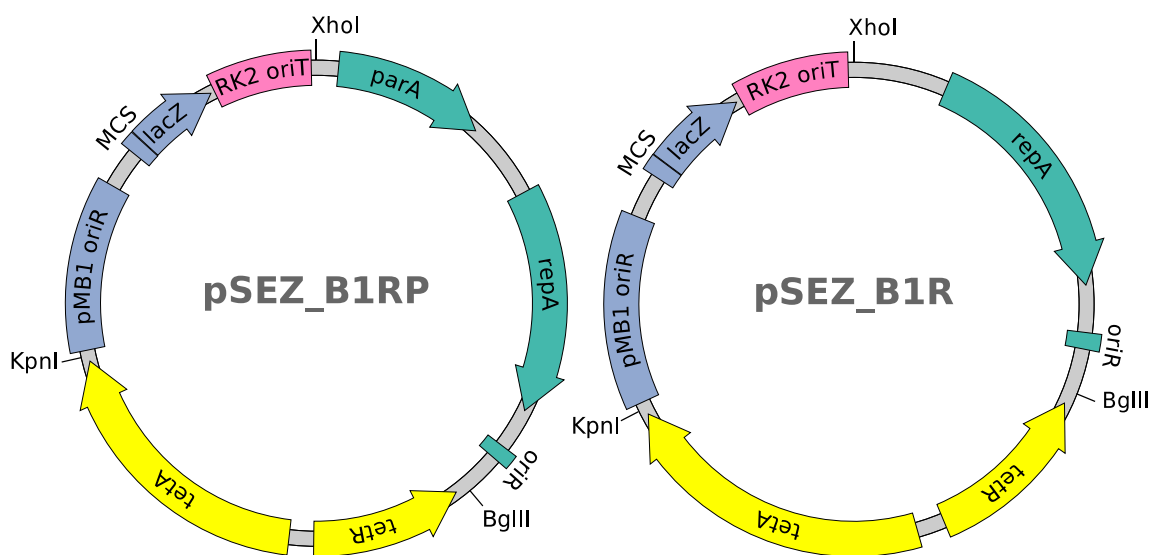
Figure 16. Final map of SEZ_B1RP and SEZ_B1R plasmids
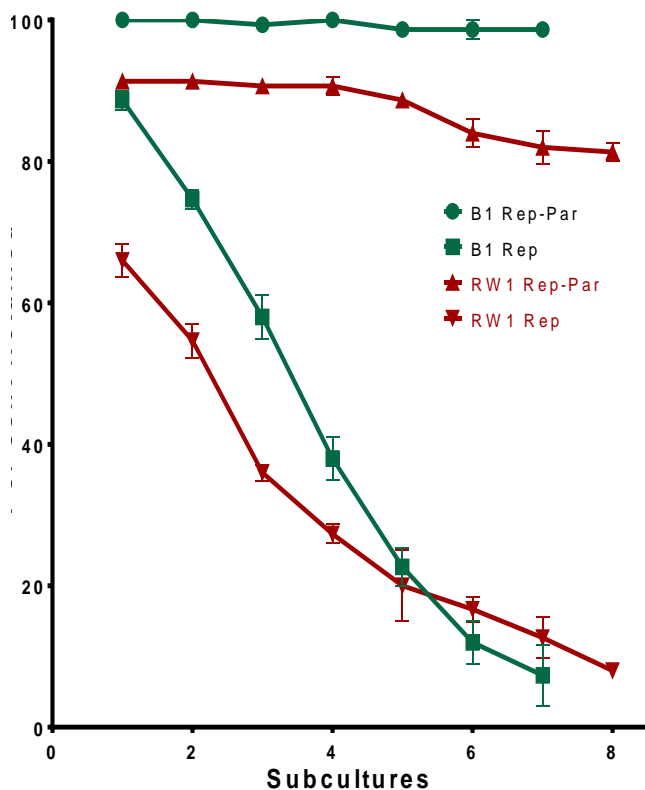
Figure 17. Stability of the new versatile *E. coli-Sphingomonas* shuttle vector after seven successive subcultures.

The pSEZ_B1RP and pSEZ_B1R vectors were mated into *S. yanoikuyae* B1 and *S. wittichii* RW1. The native plasmid pKG2 in B1 was lost on the introduction of both plasmids which was confirmed by PCR using a set of primers BlankmapF and RepAmapR. Stability tests were performed by subculturing the cells in LB medium every day and plating on selective and nonselective medium.
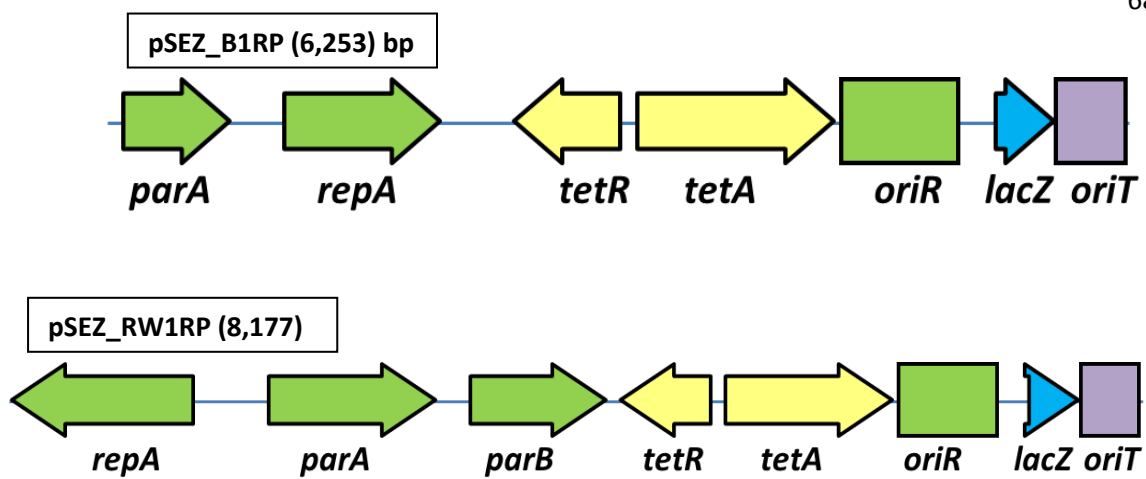
Figure 18. Genetic components of the new vectors, pSEZ_B1RP and pSEZ_RW1RP
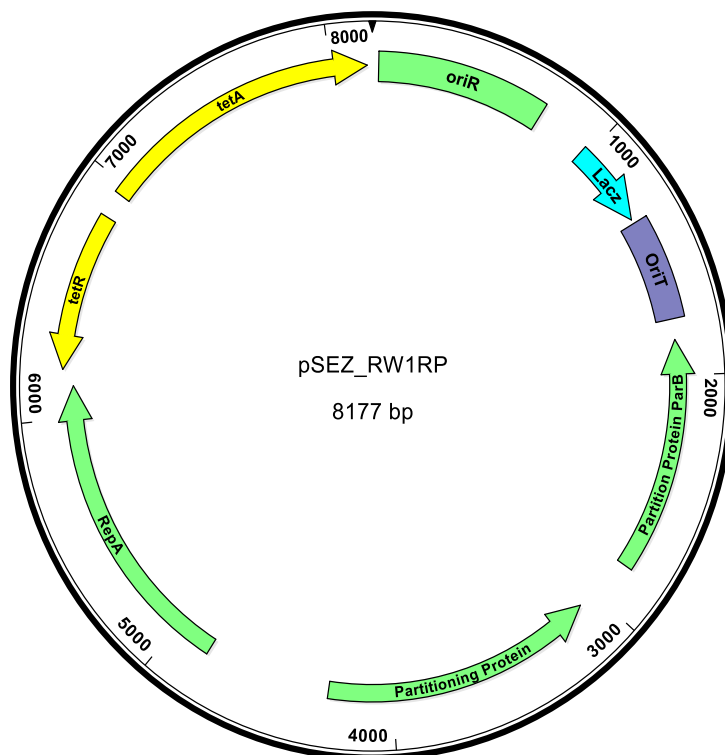
Figure 19. pSEZ_RW1RP final map

4.4. DISCUSSION

   Sphingomonas wittichii RW1 metabolizes dibenzo-*p*-dioxin and dibenzofuran as

sole carbon and energy sources. *S. wittichii* RW1 is considered a model organism that

facilitates the study of the molecular and biochemical mechanisms of dibenzo-*p*-dioxin

and other environmental toxicants metabolism. Further, intensive effort and research have

been performed to better understand the enzymes involved in the degradation pathway

and a number of these enzymes have been purified and described in detail (139, 140).

However, there is still a lack of sufficient molecular tools needed for genetic

manipulation in *Sphingomonas* to better understand the metabolism and the genes

participating in the degradation of dibenzo-*p*-dioxins as well as dibenzofurans (141). In

the current work, we aimed to develop efficient genetic tools that can be exploited to

study the metabolism of dibenzo-*p*-dioxin and dibenzofuran degradation in

Sphingomonas and to identify genes that are indispensable for growth on these

compounds. Thus, we designed and constructed a new *Sphingomonas* shuttle vector that

can be stably maintained in *Sphingomonas* species providing a useful tool for genetic

manipulation in the bacterium *S. wittichii* RW1 and for the identification and study of the

catabolic pathway genes of xenobiotic compounds such as dibenzo-*p*-dioxin and

dibenzofuran.

   The complete genome sequence of RW1 reveals that this strain, in addition to the

main chromosome, contains two circular megaplasmids pSWIT01 and pSWIT02. A

number of studies have suggested that pSWIT02 carries genes that are involved in

dibenzo-*p*-dioxin and dibenzofuran degradation. Our hypothesis is that the megaplasmid

pSWIT02 only encodes the first enzymatic step of the dibenzo-*p*-dioxin pathway.

Previous studies by other researchers suggested (erroneously) that the degradative genes of dibenzo-*p*-dioxin metabolism are expected to be located on the megaplasmid pSWIT02 rather than on the chromosome. To better clarify the role of pSWIT02 in *S. wittichii* RW1 we constructed the new plasmid pSEZ_RW1 containing the origin of replication *oriR* and associated partition (*par*) genes of pSWIT02 along with genes for tetracycline resistance. This plasmid was mated into RW1 with the goal of forcing out the megaplasmid pSWIT02 since the shared *oriR* and *par* would result in incompatible plasmids. The dual plasmid construct was subcultured multiple times in minimal medium supplemented with phenylalanine as a sole carbon source and colonies were examined for the presence of pSWIT02 by PCR. This eventually resulted in a pSWIT02 cured version of RW1. The pSEZ_RW1 plasmid is slightly unstable and loss of this plasmid was then obtained by selecting for colonies lacking tetracycline resistance. Unsurprisingly, the pSWIT02 cured strain did not grow on either dibenzo-*p*-dioxin or dibenzofuran. We used PCR to clone the *dxnA1A2*, *fdx3*, and *redA2* genes encoding a multicomponent angular dioxygenase from three different locations in pSWIT02 into the low copy number vector pRK415 so that expression of the genes is from the *lac* promoter. Moving this plasmid into the cured RW1 restored growth on dibenzo-*p*-dioxin and dibenzofuran. Growth curves on minimal medium supplemented with either compound as the sole carbon source showed that the rate and extent of growth were almost the same as the wild type strain.

Based on these experiments we conclude that the only pSWIT02 genes involved in the degradation of dibenzo-*p*-dioxin and dibenzofuran are the ones encoding the initial angular dioxygenase. This explains why very few dioxin degrading organisms are

known, that a combination of plasmid and chromosome-encoded genes are necessary for growth on this recalcitrant compound. Introducing only the two subunits of the dioxin dioxygenase (*dxnA1A2*) cloned into the unstable pRK415 vector did not restore the growth of the cured RW1 strain on dibenzo-*p*-dioxin and dibenzofuran. However, adding the genes for the electron transport components *reda2* and *fdx3* along with the genes for the dioxygenase resulted in the growth ability of RW1 on both dibenzo-*p*-dioxin and dibenzofuran. These results are consistent with previous data by Armenguad et al. (1998) who demonstrated that the activity of dioxygenase cannot be accomplished by DxnA1A2 alone and that the presence of the reductase and ferredoxin is required for the heterologous expression of the dibenzo-*p*-dioxin dioxygenase system in *E. coli* DH5α. This data is also supported by our previous work involved the generation of the dioxygenase mutant RW1ΔdxnA1 where the mutant strain was not able to grow on dibenzo-*p*-dioxin and dibenzofuran. Both these data collectively provide solid evidence that the dioxygenase enzyme consisting of the large and the small subunits is located on the megaplasmid pSWIT02 and its function is important for the growth of RW1 on dibenzo-*p*-dioxin. The dibenzo-*p*-dioxin dioxygenase was shown to exist in only one copy that is located on the megaplasmid. However, pSWIT02 genes encoding other steps in the catabolic pathway can be replaced by additional copies that are chromosomally encoded.

Growth on Dibenzofuran   Growth on Dibenzo-*p*-dioxin
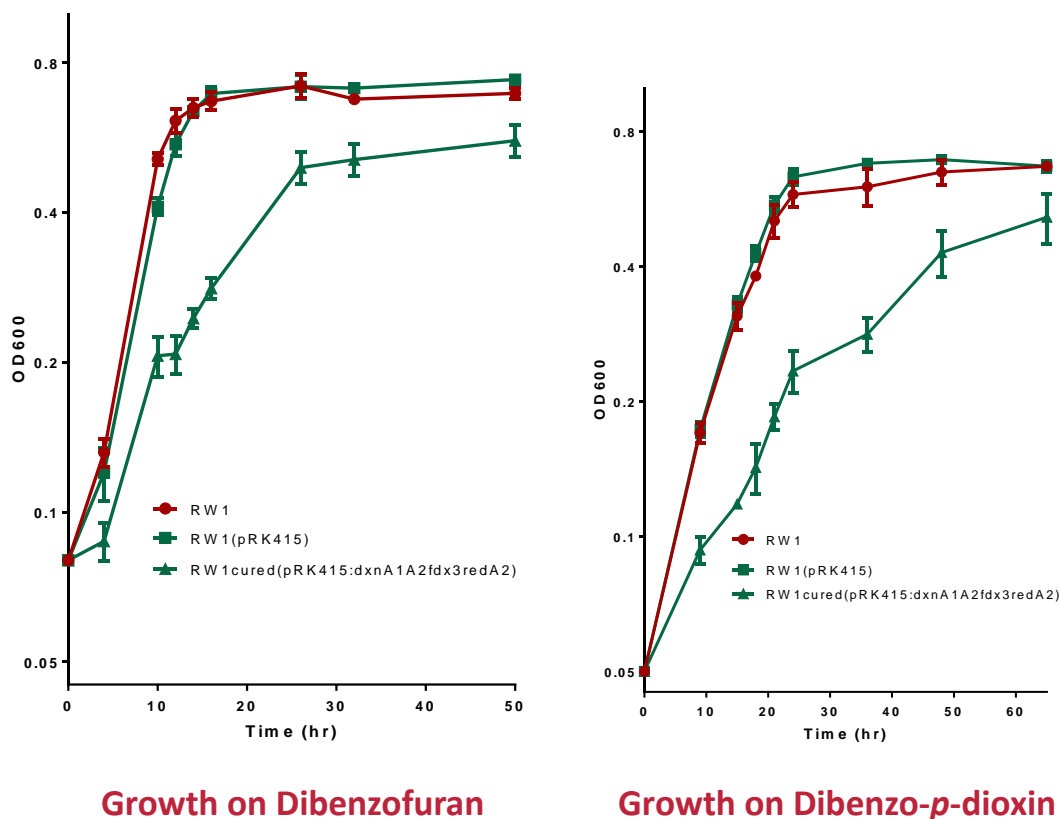
Figure 20: Complementation of the cured RW1 strain with the plasmid pRK415 carrying the initial dioxygenase genes (DxnA1A2, RedA2 and RedA3)

The broad host range plasmid pRK415 carrying the four genes, DxnA1A2, RedA2 ,and Fdx3, was moved into the cured RW1 via triparental mating method. The resulting transconjugant and the wild type strain (control strain) were then grown in MSB broth medium supplemented with 3 mM dibenzofuran and 3 mM dibenzo-p-dioxin. The growth turbidity was measured every 2 hours for dibenzofuran growth culture and every 3 hours for dioxin growth culture. The growth curve results showed that the rate of the growth of the cured RWI with only dioxygenase genes expressed on plasmid pRK415 was almost

the same as the growth of the wild type strain. These results prove that the dioxin

dioxygenase components are the only required genes located on the megaplasmid for

dioxin and dibenzofuran metabolism.

REFERENCES

1. Zeiser J, Muhlenbeck LH, Schweiger P, Deppenmeier U. 2014. Characterization of a periplasmic quinoprotein from *Sphingomonas wittichii* that functions as aldehyde dehydrogenase. Appl Microbiol Biotechnol 98:2067-79.

2. Basta T, Buerger S, Stolz A. 2005. Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. Microbiology 151:2025-37.

3. Gatheru Waigi M, Sun K, Gao Y. 2017. Sphingomonads in microbe-assisted phytoremediation: tackling soil pollution. Trends Biotechnol 35:883-899.

4. Kim E, Aversano PJ, Romine MF, Schneider RP, Zylstra GJ. 1996. Homology between genes for aromatic hydrocarbon degradation in surface and deep-subsurface *Sphingomonas* strains. Appl Environ Microbiol 62:1467-70.

5. Aylward FO, McDonald BR, Adams SM, Valenzuela A, Schmidt RA, Goodwin LA, Woyke T, Currie CR, Suen G, Poulsen M. 2013. Comparison of 26 sphingomonad genomes reveals diverse environmental adaptations and biodegradative capabilities. Appl Environ Microbiol 79:3724-33.

6. Barry KP, Taylor EA. 2013. Characterizing the promiscuity of LigAB, a lignin catabolite degrading extradiol dioxygenase from *Sphingomonas paucimobilis* SYK-6. Biochemistry 52:6724-36.

7. Mnich E, Vanholme R, Oyarce P, Liu S, Lu F, Goeminne G, Jorgensen B, Motawie MS, Boerjan W, Ralph J, Ulvskov P, Moller BL, Bjarnholt N, Harholt J. 2017. Degradation of lignin beta-aryl ether units in *Arabidopsis thaliana*

expressing LigD, LigF and LigG from *Sphingomonas paucimobilis* SYK-6. Plant Biotechnol J 15:581-593.

8. Schultheis E, Dreger MA, Nimtz M, Wray V, Hempel DC, Nortemann B. 2008. Structural characterization of the exopolysaccharide PS-EDIV from *Sphingomonas pituitosa* strain DSM 13101. Appl Microbiol Biotechnol 78:1017-24.

9. Asker D, Beppu T, Ueda K. 2007. *Sphingomonas astaxanthinifaciens* sp. nov., a novel astaxanthin-producing bacterium of the family Sphingomonadaceae isolated from Misasa, Tottori, Japan. FEMS Microbiol Lett 273:140-8.

10. Seo EJ, Yoo SH, Oh KW, Cha J, Lee HG, Park CS. 2004. Isolation of an exopolysaccharide-producing bacterium, *Sphingomonas* sp. CS101, which forms an unusual type of sphingan. Biosci Biotechnol Biochem 68:1146-8.

11. Ying XX, Ping Z, Sha L, Ye CX, Zhong Y, Hong X. 2014. Production of rhamsan gum using a two-stage pH control strategy by *Sphingomonas* sp. CGMCC 6833. Appl Biochem Biotechnol 172:168-75.

12. Zhu L, Wu X, Li O, Qian C, Gao H. 2012. Cloning and characterization of genes involved in nostoxanthin biosynthesis of *Sphingomonas elodea* ATCC 31461. PLoS One 7:e35099.

13. Tokajian S, Issa N, Salloum T, Ibrahim J, Farah M. 2016. 16S-23S rRNA gene intergenic spacer region variability helps resolve closely related sphingomonads. Front Microbiol 7:149.

14. Nielsen TK, Carstens AB, Browne P, Lametsch R, Neve H, Kot W, Hansen LH. 2017. The first characterized phage against a member of the ecologically

important sphingomonads reveals high dissimilarity against all other known phages. Sci Rep 7:13566.

15.   Tiirola MA, Wang H, Paulin L, Kulomaa MS. 2002. Evidence for natural horizontal transfer of the *pcpB* gene in the evolution of polychlorophenol-degrading sphingomonads. Appl Environ Microbiol 68:4495-501.

16.   Chaudhary DK, Kim J. 2017. *Sphingomonas olei* sp. nov., with the ability to degrade aliphatic hydrocarbons, isolated from oil-contaminated soil. Int J Syst Evol Microbiol 67:2731-2738.

17.   Takeuchi M, Hamana K, Hiraishi A. 2001. Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. Int J Syst Evol Microbiol 51:1405-17.

18.   Yabuuchi E, Kosako Y, Fujiwara N, Naka T, Matsunaga I, Ogura H, Kobayashi K. 2002. Emendation of the genus *Sphingomonas* Yabuuchi et al. 1990 and junior objective synonymy of the species of three genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, in conjunction with *Blastomonas ursincola*. Int J Syst Evol Microbiol 52:1485-96.

19.   Takeuchi M, Sakane T, Yanagi M, Yamasato K, Hamana K, Yokota A. 1995. Taxonomic study of bacteria isolated from plants: proposal of *Sphingomonas rosa* sp. nov., *Sphingomonas pruni* sp. nov., *Sphingomonas asaccharolytica* sp. nov., and *Sphingomonas mali* sp. nov. Int J Syst Bacteriol 45:334-41.

20.   Yun SS, Siddiqi MZ, Lee SY, Kim MS, Choi K, Im WT. 2016. *Sphingomonas hankyongensis* sp. nov. isolated from tap water. Arch Microbiol 198:767-71.

21. Park HK, Han JH, Kim TS, Joung Y, Cho SH, Kwon SW, Kim SB. 2015. *Sphingomonas aeria* sp. nov. from indoor air of a pharmaceutical environment. Antonie Van Leeuwenhoek 107:47-53.

22. Hoang VA, Kim YJ, Nguyen NL, Yang DC. 2012. *Sphingomonas ginsengisoli* sp. nov., isolated from soil of a ginseng field. J Gen Appl Microbiol 58:421-8.

23. Lee JH, Kim DI, Choe HN, Lee SD, Seong CN. 2017. *Sphingomonas limnosediminicola* sp. nov. and *Sphingomonas palustris* sp. nov., isolated from freshwater environments. Int J Syst Evol Microbiol 67:2834-2841.

24. Del Borgo C, Maneschi F, Belvisi V, Morelli F, Vetica A, Marocco R, Tieghi T, Lichtner M, Mastroianni CM. 2013. Postpartum fever in the presence of a fibroid: *Sphingomonas paucimobilis* sepsis associated with pyomyoma. BMC Infect Dis 13:574.

25. Tai ML, Velayuthan RD. 2014. *Sphingomonas paucimobilis*: an unusual cause of meningitis-case report. Neurol Med Chir (Tokyo) 54:337-40.

26. Gulati P, Ghosh M. 2017. Biofilm forming ability of *Sphingomonas paucimobilis* isolated from community drinking water systems on plumbing materials used in water distribution. J Water Health 15:942-954.

27. Chen Q, Wang CH, Deng SK, Wu YD, Li Y, Yao L, Jiang JD, Yan X, He J, Li SP. 2014. Novel three-component Rieske non-heme iron oxygenase system catalyzing the N-dealkylation of chloroacetanilide herbicides in sphingomonads DC-6 and DC-2. Appl Environ Microbiol 80:5078-85.

28. Yabuuchi E, Yano I, Oyaizu H, Hashimoto Y, Ezaki T, Yamamoto H. 1990. Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov.,

Sphingomonas parapaucimobilis sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. Microbiol Immunol 34:99-119.

29. Hashimoto W, Murata K. 1998. Alpha-L-rhamnosidase of *Sphingomonas* sp. R1 producing an unusual exopolysaccharide of sphingan. Biosci Biotechnol Biochem 62:1068-74.

30. Ahn JH, Kim BC, Kim SJ, Lee GH, Song J, Kwon SW, Weon HY. 2015. *Sphingomonas parvus* sp. nov. isolated from a ginseng-cultivated soil. J Microbiol 53:673-7.

31. Yabuuchi E, Yamamoto H, Terakubo S, Okamura N, Naka T, Fujiwara N, Kobayashi K, Kosako Y, Hiraishi A. 2001. Proposal of *Sphingomonas wittichii* sp. nov. for strain RW1T, known as a dibenzo-*p*-dioxin metabolizer. Int J Syst Evol Microbiol 51:281-92.

32. Hashidoko Y, Kitagawa E, Iwahashi H, Purnomo E, Hasegawa T, Tahara S. 2007. Design of sphingomonad-detecting probes for a DNA array, and its application to investigate the behavior, distribution, and source of *Rhizospherous sphingomonas* and other sphingomonads inhabiting an acid sulfate soil paddock in Kalimantan, Indonesia. Biosci Biotechnol Biochem 71:343-51.

33. Zhou L, Li H, Zhang Y, Han S, Xu H. 2014. Development of genus-specific primers for better understanding the diversity and population structure of *Sphingomonas* in soils. J Basic Microbiol 54:880-8.

34.   Lee J, Shin SC, Kim SJ, Kim BK, Hong SG, Kim EH, Park H, Lee H. 2012. Draft
        genome sequence of a *Sphingomonas* sp., an endosymbiotic bacterium isolated
        from an arctic lichen *Umbilicaria* sp. J Bacteriol 194:3010-1.

35.   Lee H, Kim DU, Lee S, Yun J, Park S, Yoon JH, Park SY, Ka JO. 2017.
        Sphingomonas carri sp. nov., isolated from a car air-conditioning system. Int J
        Syst Evol Microbiol 67:4069-4074.

36.   Chen H, Jogler M, Rohde M, Klenk HP, Busse HJ, Tindall BJ, Sproer C,
        Overmann J. 2012. Reclassification and emended description of *Caulobacter
        leidyi* as *Sphingomonas leidyi* comb. nov., and emendation of the genus
        Sphingomonas. Int J Syst Evol Microbiol 62:2835-43.

37.   Schuler L, Ni Chadhain SM, Jouanneau Y, Meyer C, Zylstra GJ, Hols P, Agathos
        SN. 2008. Characterization of a novel angular dioxygenase from fluorene-
        degrading *Sphingomonas* sp. strain LB126. Appl Environ Microbiol 74:1050-7.

38.   Gan HM, Gan HY, Ahmad NH, Aziz NA, Hudson AO, Savka MA. 2014. Whole
        genome sequencing and analysis reveal insights into the genetic structure,
        diversity and evolutionary relatedness of luxI and *luxR* homologs in bacteria
        belonging to the Sphingomonadaceae family. Front Cell Infect Microbiol 4:188.

39.   Demaneche S, Meyer C, Micoud J, Louwagie M, Willison JC, Jouanneau Y.
        2004. Identification and functional analysis of two aromatic-ring-hydroxylating
        dioxygenases from a sphingomonas strain that degrades various polycyclic
        aromatic hydrocarbons. Appl Environ Microbiol 70:6714-25.

40. Aly HA, Huu NB, Wray V, Junca H, Pieper DH. 2008. Two angular dioxygenases contribute to the metabolic versatility of dibenzofuran-degrading *Rhodococcus* sp. strain HA01. Appl Environ Microbiol 74:3812-22.

41. Armengaud J, Happe B, Timmis KN. 1998. Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J Bacteriol 180:3954-66.

42. Halden RU, Colquhoun DR, Wisniewski ES. 2005. Identification and phenotypic characterization of *Sphingomonas wittichii* strain RW1 by peptide mass fingerprinting using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 71:2442-51.

43. Colquhoun DR, Hartmann EM, Halden RU. 2012. Proteomic profiling of the dioxin-degrading bacterium *Sphingomonas wittichii* RW1. J Biomed Biotechnol 2012:408690.

44. Halden RU. 2006. Comment on "biological removal of polychlorinated dibenzo-p-dioxins from incinerator fly ash by *Sphingomonas wittichii* RW1" by I.-H. Nam, Y.-M. Kim, B.-H. Kim, K. Murugesan, and Y.-S. Chang. Water Res 40:1918-20.

45. Hong HB, Chang YS, Nam IH, Fortnagel P, Schmidt S. 2002. Biotransformation of 2,7-dichloro- and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* RW1. Appl Environ Microbiol 68:2584-8.

46. Wilkes H, Wittich R, Timmis KN, Fortnagel P, Francke W. 1996. Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. Appl Environ Microbiol 62:367-71.

47. Stolz A. 2009. Molecular characteristics of xenobiotic-degrading sphingomonads. Appl Microbiol Biotechnol 81:793-811.

48. Wittich RM, Wilkes H, Sinnwell V, Francke W, Fortnagel P. 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl Environ Microbiol 58:1005-10.

49. Yun NR, Shin YK, Hwang SY, Kuraishi H, Sugiyama J. 2000. Chemotaxonomic and phylogenetic analyses of *Sphingomonas* strains isolated from ears of plants in the family *Gramineae* and a proposal of *Sphingomonas roseoflava* sp. nov. J Gen Appl Microbiol 46:9-18.

50. Aso Y, Miyamoto Y, Harada KM, Momma K, Kawai S, Hashimoto W, Mikami B, Murata K. 2006. Engineered membrane superchannel improves bioremediation potential of dioxin-degrading bacteria. Nat Biotechnol 24:188-9.

51. Miller TR, Delcher AL, Salzberg SL, Saunders E, Detter JC, Halden RU. 2010. Genome sequence of the dioxin-mineralizing bacterium *Sphingomonas wittichii* RW1. J Bacteriol 192:6101-2.

52. Happe B, Eltis LD, Poth H, Hedderich R, Timmis KN. 1993. Characterization of 2,2',3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-*p*-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. J Bacteriol 175:7313-20.

53. Desai AM, Autenrieth RL, Dimitriou-Christidis P, McDonald TJ. 2008. Biodegradation kinetics of select polycyclic aromatic hydrocarbon (PAH) mixtures by *Sphingomonas paucimobilis* EPA505. Biodegradation 19:223-33.

54. Ruffini Castiglione M, Giorgetti L, Becarelli S, Siracusa G, Lorenzi R, Di Gregorio S. 2016. Polycyclic aromatic hydrocarbon-contaminated soils: bioaugmentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of Vicia faba L. Environ Sci Pollut Res Int 23:7930-41.

55. Rybicki BA, Nock NL, Savera AT, Tang D, Rundle A. 2006. Polycyclic aromatic hydrocarbon-DNA adduct formation in prostate carcinogenesis. Cancer Lett 239:157-67.

56. Alegbeleye OO, Opeolu BO, Jackson V. 2017. Bioremediation of polycyclic aromatic hydrocarbon (PAH) compounds: (acenaphthene and fluorene) in water using indigenous bacterial species isolated from the Diep and Plankenburg rivers, Western Cape, South Africa. Braz J Microbiol 48:314-325.

57. Pinyakong O, Habe H, Supaka N, Pinpanichkarn P, Juntongjin K, Yoshida T, Furihata K, Nojiri H, Yamane H, Omori T. 2000. Identification of novel metabolites in the degradation of phenanthrene by *Sphingomonas* sp. strain P2. FEMS Microbiol Lett 191:115-21.

58. Ashida H. 2000. Suppressive effects of flavonoids on dioxin toxicity. Biofactors 12:201-6.

59. Boyd SA, Sallach JB, Zhang Y, Crawford R, Li H, Johnston CT, Teppen BJ, Kaminski NE. 2017. Sequestration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by activated carbon eliminates bioavailability and the suppression of immune function in mice. Environ Toxicol Chem 36:2671-2678.

60. Kleinhenz S, Jira W, Schwind KH. 2006. Dioxin and polychlorinated biphenyl analysis: automation and improvement of clean-up established by example of spices. Mol Nutr Food Res 50:362-7.

61. Yakovleva L, Handy CJ, Yagi H, Sayer JM, Jerina DM, Shuman S. 2006. Intercalating polycyclic aromatic hydrocarbon-DNA adducts poison DNA religation by *Vaccinia* topoisomerase and act as roadblocks to digestion by exonuclease III. Biochemistry 45:7644-53.

62. Arfmann H, Timmis KN, Wittich R. 1997. Mineralization of 4-chlorodibenzofuran by a consortium consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. Appl Environ Microbiol 63:3458-62.

63. Adrion AC, Singleton DR, Nakamura J, Shea D, Aitken MD. 2016. Improving polycyclic aromatic hydrocarbon biodegradation in contaminated soil through low-level surfactant addition after conventional bioremediation. Environ Eng Sci 33:659-670.

64. Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. 2006. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicol Sci 92:526-36.

65. Re N, Kataoka VM, Cardoso CA, Alcantara GB, de Souza JB. 2015. Polycyclic aromatic hydrocarbon concentrations in gas and particle phases and source determination in atmospheric samples from a semiurban area of Dourados, Brazil. Arch Environ Contam Toxicol 69:69-80.

66. Adekunle AS, Oyekunle JAO, Ojo OS, Maxakato NW, Olutona GO, Obisesan OR. 2017. Determination of polycyclic aromatic hydrocarbon levels of groundwater in Ife north local government area of Osun state, Nigeria. Toxicol Rep 4:39-48.

67. Nam IH, Kim YM, Schmidt S, Chang YS. 2006. Biotransformation of 1,2,3-tri- and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* strain RW1. Appl Environ Microbiol 72:112-6.

68. Chaudhary P, Sharma R, Singh SB, Nain L. 2011. Bioremediation of PAH by *Streptomyces* sp. Bull Environ Contam Toxicol 86:268-71.

69. Chauhan A, Fazlurrahman, Oakeshott JG, Jain RK. 2008. Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. Indian J Microbiol 48:95-113.

70. Zhao Q, Yue S, Bilal M, Hu H, Wang W, Zhang X. 2017. Comparative genomic analysis of 26 *Sphingomonas* and *Sphingobium* strains: dissemination of bioremediation capabilities, biodegradation potential and horizontal gene transfer. Sci Total Environ 609:1238-1247.

71. Willison JC. 2004. Isolation and characterization of a novel sphingomonad capable of growth with chrysene as sole carbon and energy source. FEMS Microbiol Lett 241:143-50.

72. Kanaly RA, Harayama S. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059-67.

73. Kanaly RA, Harayama S. 2010. Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. Microb Biotechnol 3:136-64.

74. Pinyakong O, Habe H, Kouzuma A, Nojiri H, Yamane H, Omori T. 2004. Isolation and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase from acenaphthene and acenaphthylene degrading *Sphingomonas* sp. strain A4. FEMS Microbiol Lett 238:297-305.

75. Beliaeff B, O'Connor TP, Munschy C, Raffin B, Claisse D. 2002. Comparison of polycyclic aromatic hydrocarbon levels in mussels and oysters in France and the United States. Environ Toxicol Chem 21:1783-7.

76. Bisht S, Pandey P, Bhargava B, Sharma S, Kumar V, Sharma KD. 2015. Bioremediation of polyaromatic hydrocarbons (PAHs) using rhizosphere technology. Braz J Microbiol 46:7-21.

77. Davie-Martin CL, Stratton KG, Teeguarden JG, Waters KM, Simonich SLM. 2017. Implications of bioremediation of polycyclic aromatic hydrocarbon-contaminated soils for human health and cancer risk. Environ Sci Technol 51:9458-9468.

78. Ni Chadhain SM, Norman RS, Pesce KV, Kukor JJ, Zylstra GJ. 2006. Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. Appl Environ Microbiol 72:4078-87.

79. Ashok BT, Saxena S, Musarrat J. 1995. Isolation and characterization of four polycyclic aromatic hydrocarbon degrading bacteria from soil near an oil refinery. Lett Appl Microbiol 21:246-8.

80. Simarro R, Gonzalez N, Bautista LF, Molina MC. 2013. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by a wood-degrading consortium at low temperatures. FEMS Microbiol Ecol 83:438-49.

81. Zylstra GJ, Wang XP, Kim E, Didolkar VA. 1994. Cloning and analysis of the genes for polycyclic aromatic hydrocarbon degradation. Ann N Y Acad Sci 721:386-98.

82. Muangchinda C, Pansri R, Wongwongsee W, Pinyakong O. 2013. Assessment of polycyclic aromatic hydrocarbon biodegradation potential in mangrove sediment from Don Hoi Lot, Samut Songkram Province, Thailand. J Appl Microbiol 114:1311-24.

83. Garcia de Llasera MP, Olmos-Espejel Jde J, Diaz-Flores G, Montano-Montiel A. 2016. Biodegradation of benzo(a)pyrene by two freshwater microalgae *Selenastrum capricornutum* and *Scenedesmus acutus*: a comparative study useful for bioremediation. Environ Sci Pollut Res Int 23:3365-75.

84. Hesham Ael L, Mawad AM, Mostafa YM, Shoreit A. 2014. Biodegradation ability and catabolic genes of petroleum-degrading *Sphingomonas koreensis* strain ASU-06 isolated from Egyptian oily soil. Biomed Res Int 2014:127674.

85. Kasuga K, Nitta A, Kobayashi M, Habe H, Nojiri H, Yamane H, Omori T, Kojima I. 2013. Cloning of *dfdA* genes from *Terrabacter* sp. strain DBF63 encoding dibenzofuran 4,4a-dioxygenase and heterologous expression in *Streptomyces lividans*. Appl Microbiol Biotechnol 97:4485-98.

86. Wittich RM. 1998. Degradation of dioxin-like compounds by microorganisms. Appl Microbiol Biotechnol 49:489-99.

87. Cunliffe M, Kertesz MA. 2006. Autecological properties of soil sphingomonads involved in the degradation of polycyclic aromatic hydrocarbons. Appl Microbiol Biotechnol 72:1083-9.

88. Abbasian F, Lockington R, Megharaj M, Naidu R. 2016. A review on the genetics of aliphatic and aromatic hydrocarbon degradation. Appl Biochem Biotechnol 178:224-50.

89. Yan X, Gu T, Yi Z, Huang J, Liu X, Zhang J, Xu X, Xin Z, Hong Q, He J, Spain JC, Li S, Jiang J. 2016. Comparative genomic analysis of isoproturon-mineralizing sphingomonads reveals the isoproturon catabolic mechanism. Environ Microbiol 18:4888-4906.

90. Baboshin M, Akimov V, Baskunov B, Born TL, Khan SU, Golovleva L. 2008. Conversion of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. VKM B-2434. Biodegradation 19:567-76.

91. Baldwin BR, Nakatsu CH, Nies L. 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ Microbiol 69:3350-8.

92. Nam IH, Kim YM, Schmidt S, Chang YS. 2006. Biotransformation of 1,2,3-tri- and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* strain RW1. Appl Environ Microbiol 72:112-6.

93. Peng P, Yang H, Jia R, Li L. 2013. Biodegradation of dioxin by a newly isolated *Rhodococcus* sp. with the involvement of self-transmissible plasmids. Appl Microbiol Biotechnol 97:5585-95.

94. Brzuzy LP, Hites RA. 1995. Estimating the atmospheric deposition of polychlorinated dibenzo-*p*-dioxins and dibenzofurans from soils. Environ Sci Technol 29:2090-8.

95. Iida T, Mukouzaka Y, Nakamura K, Kudo T. 2002. Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran degradation by *Terrabacter* sp. strain YK3. Appl Environ Microbiol 68:3716-23.

96. Eskenazi B, Warner M, Sirtori M, Fuerst T, Rauch SA, Brambilla P, Mocarelli P, Rubinacci A. 2014. Serum dioxin concentrations and bone density and structure in the Seveso Women's Health Study. Environ Health Perspect 122:51-7.

97. Bunz PV, Schmidt S. 1997. The microbial degradation of halogenated diaryl ethers. Biotechnol Adv 15:621-32.

98. Linden J, Korkalainen M, Lensu S, Tuomisto J, Pohjanvirta R. 2005. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and leptin on hypothalamic mRNA expression of factors participating in food intake regulation in a TCDD-sensitive and a TCDD-resistant rat strain. J Biochem Mol Toxicol 19:139-48.

99. Calkosinski I, Rosinczuk-Tonderys J, Bazan J, Dzierzba K, Calkosinska M, Majda J, Dobrzynski M, Bronowicka-Szydelko A. 2013. The influence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on hematological parameters during experimentally induced pleuritis in rats. Inflammation 36:387-404.

100. Ju Q, Fimmel S, Hinz N, Stahlmann R, Xia L, Zouboulis CC. 2011. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin alters sebaceous gland cell differentiation in vitro. Exp Dermatol 20:320-5.

101. Astroff B, Zacharewski T, Safe S, Arlotto MP, Parkinson A, Thomas P, Levin W. 1988. 6-Methyl-1,3,8-trichlorodibenzofuran as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist: inhibition of the induction of rat cytochrome P-450 isozymes and related monooxygenase activities. Mol Pharmacol 33:231-6.

102. Bowers OJ, Sommersted KB, Sowell RT, Boling GE, Hanneman WH, Titus RG, Dekrey GK. 2006. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) reduces Leishmania major burdens in C57BL/6 mice. Am J Trop Med Hyg 75:749-52.

103. Bokare V, Murugesan K, Kim JH, Kim EJ, Chang YS. 2012. Integrated hybrid treatment for the remediation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Sci Total Environ 435-436:563-6.

104. Abbott BD, Buckalew AR, Leffler KE. 2005. Effects of epidermal growth factor (EGF), transforming growth factor-alpha (TGFalpha), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on fusion of embryonic palates in serum-free organ culture using wild-type, EGF knockout, and TGFalpha knockout mouse strains. Birth Defects Res A Clin Mol Teratol 73:447-54.

105. Ivnitski I, Elmaoued R, Walker MK. 2001. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) inhibition of coronary development is preceded by a decrease in myocyte proliferation and an increase in cardiac apoptosis. Teratology 64:201-12.

106. National Toxicology P. 2011. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. Rep Carcinog 12:396-8.

107. Zhang H, Jiang X, Lu L, Xiao W. 2015. Biodegradation of polychlorinated biphenyls (PCBs) by the novel identified cyanobacterium *Anabaena* PD-1. PLoS One 10:e0131450.

108. Amakura Y, Tsutsumi T, Nakamura M, Kitagawa H, Fujino J, Sasaki K, Toyoda M, Yoshida T, Maitani T. 2003. Activation of the aryl hydrocarbon receptor by some vegetable constituents determined using in vitro reporter gene assay. Biol Pharm Bull 26:532-9.

109. Cespedes MA, Galindo MI, Couso JP. 2010. Dioxin toxicity in vivo results from an increase in the dioxin-independent transcriptional activity of the aryl hydrocarbon receptor. PLoS One 5:e15382.

110. Camacho IA, Singh N, Hegde VL, Nagarkatti M, Nagarkatti PS. 2005. Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. J Immunol 175:90-103.

111. Amenya HZ, Tohyama C, Ohsako S. 2016. Dioxin induces Ahr-dependent robust DNA demethylation of the Cyp1a1 promoter via Tdg in the mouse liver. Sci Rep 6:34989.

112. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature 453:106-9.

113. Alaluusua S, Calderara P, Gerthoux PM, Lukinmaa PL, Kovero O, Needham L, Patterson DG, Jr., Tuomisto J, Mocarelli P. 2004. Developmental dental aberrations after the dioxin accident in Seveso. Environ Health Perspect 112:1313-8.

114. Collier A, Orr L, Morris J, Blank J. 2008. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the mortality and growth of two amphibian species (*Xenopus laevis* and *Pseudacris triseriata*). Int J Environ Res Public Health 5:368-77.

115. Dhanabalan S, D'Cruz S C, Mathur PP. 2010. Effects of corticosterone and 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin on epididymal antioxidant system in adult rats. J Biochem Mol Toxicol 24:242-9.

116. Harvey WA, Jurgensen K, Pu X, Lamb CL, Cornell KA, Clark RJ, Klocke C, Mitchell KA. 2016. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases human hepatic stellate cell activation. Toxicology 344-346:26-33.

117. Alkorta I, Epelde L, Garbisu C. 2017. Environmental parameters altered by climate change affect the activity of soil microorganisms involved in bioremediation. FEMS Microbiol Lett 364.

118. Mydy LS, Mashhadi Z, Knight TW, Fenske T, Hagemann T, Hoppe RW, Han L, Miller TR, Schwabacher AW, Silvaggi NR. 2017. Swit_4259, an acetoacetate decarboxylase-like enzyme from *Sphingomonas wittichii* RW1. Acta Crystallogr F Struct Biol Commun 73:672-681.

119. Leys NM, Bastiaens L, Verstraete W, Springael D. 2005. Influence of the carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by *Mycobacterium* and *Sphingomonas* in soil. Appl Microbiol Biotechnol 66:726-36.

120. Pan T, Deng T, Zeng X, Dong W, Yu S. 2016. Extractive biodegradation and bioavailability assessment of phenanthrene in the cloud point system by *Sphingomonas polyaromaticivorans*. Appl Microbiol Biotechnol 100:431-7.

121. Deshmukh R, Khardenavis AA, Purohit HJ. 2016. Diverse metabolic capacities of fungi for bioremediation. Indian J Microbiol 56:247-64.

122. Uchimura H, Horisaki T, Umeda T, Noguchi H, Usami Y, Li L, Terada T, Nakamura S, Shimizu K, Takemura T, Habe H, Furihata K, Omori T, Yamane H, Nojiri H. 2008. Alteration of the substrate specificity of the angular dioxygenase carbazole 1,9a-dioxygenase. Biosci Biotechnol Biochem 72:3237-48.

123. Kaczmarczyk A, Vorholt JA, Francez-Charlot A. 2012. Markerless gene deletion system for sphingomonads. Appl Environ Microbiol 78:3774-7.

124. Gibson DT, Roberts RL, Wells MC, Kobal VM. 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem Biophys Res Commun 50:211-9.

125. Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 43:159-271.

126. Dennis JJ, Zylstra GJ. 1998. Improved antibiotic-resistance cassettes through restriction site elimination using Pfu DNA polymerase PCR. Biotechniques 25:772-4, 776.

127. Dennis JJ, Zylstra GJ. 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl Environ Microbiol 64:2710-5.

128. Tang PW, Chua PS, Chong SK, Mohamad MS, Choon YW, Deris S, Omatu S, Corchado JM, Chan WH, Rahim RA. 2015. A review of gene knockout strategies for microbial cells. Recent Pat Biotechnol 9:176-97.

129. Hamilton CM, Aldea M, Washburn BK, Babitzke P, Kushner SR. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J Bacteriol 171:4617-22.

130. Nakashima N, Miyazaki K. 2014. Bacterial cellular engineering by genome editing and gene silencing. Int J Mol Sci 15:2773-93.

131. Basta T, Keck A, Klein J, Stolz A. 2004. Detection and characterization of conjugative degradative plasmids in xenobiotic-degrading *Sphingomonas* strains. J Bacteriol 186:3862-72.

132. Barton BM, Harding GP, Zuccarelli AJ. 1995. A general method for detecting and sizing large plasmids. Anal Biochem 226:235-40.

133. Blanca-Ordonez H, Oliva-Garcia JJ, Perez-Mendoza D, Soto MJ, Olivares J, Sanjuan J, Nogales J. 2010. pSymA-dependent mobilization of the *Sinorhizobium meliloti* pSymB megaplasmid. J Bacteriol 192:6309-12.

134. Botelho J, Grosso F, Quinteira S, Mabrouk A, Peixe L. 2017. The complete nucleotide sequence of an IncP-2 megaplasmid unveils a mosaic architecture comprising a putative novel blaVIM-2-harbouring transposon in *Pseudomonas aeruginosa*. J Antimicrob Chemother 72:2225-2229.

135. Urata M, Uchimura H, Noguchi H, Sakaguchi T, Takemura T, Eto K, Habe H, Omori T, Yamane H, Nojiri H. 2006. Plasmid pCAR3 contains multiple gene sets

involved in the conversion of carbazole to anthranilate. Appl Environ Microbiol 72:3198-3205.

136. Stolz A. 2014. Degradative plasmids from sphingomonads. FEMS Microbiol Lett 350:9-19.

137. Hartmann EM, Badalamenti JP, Krajmalnik-Brown R, Halden RU. 2012. Quantitative PCR for tracking the megaplasmid-borne biodegradation potential of a model sphingomonad. Appl Environ Microbiol 78:4493-6.

138. Shintani M, Urata M, Inoue K, Eto K, Habe H, Omori T, Yamane H, Nojiri H. 2007. The *Sphingomonas* plasmid pCAR3 is involved in complete mineralization of carbazole. J Bacteriol 189:2007-20.

139. Seah SY, Ke J, Denis G, Horsman GP, Fortin PD, Whiting CJ, Eltis LD. 2007. Characterization of a C-C bond hydrolase from *Sphingomonas wittichii* RW1 with novel specificities towards polychlorinated biphenyl metabolites. J Bacteriol 189:4038-45.

140. Hartmann EM, Armengaud J. 2014. Shotgun proteomics suggests involvement of additional enzymes in dioxin degradation by *Sphingomonas wittichii* RW1. Environ Microbiol 16:162-76.

141. Ochou M, Saito M, Kurusu Y. 2008. Characterization of two compatible small plasmids from *Sphingobium yanoikuyae*. Biosci Biotechnol Biochem 72:1130-3.