Cloning and Expression of Three Extradiol Dioxygenases genes Type I from the Unique Dioxin Degrader *Sphingomonas Wittichii* RW1

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Abstract: *Sphingomonas wittichii* RW1 is the unique dioxin degrader bacterium, where dioxin compounds is the most problematic environmental pollutants which cause cancer and a number of serious effects on the immune system. Extradiol dioxygenases (Edos) play a pivotal role in the metabolism of dioxin degradation. Three Edos type I genes termed *edo9, edo10* and *edo11* were identified and characterized in *Sphingomonas wittichii* RW1. The three genes were amplified and cloned into pTz57R/T Vector, all the three clones harbor *edo9, edo10*, and *edo11* capable to *meta*-cleave 2,3-dihydroxybiphenyl turned into yellow coloration compound 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoate.. Expression of the three genes *edo9, edo10,* or edo11 was observed by SDS PAGE at 40KDa, 35KDa and 36KDa, respectively. These genes may act as functional genes in the metabolic pathway of the Dioxin. These findings may benefit in the bioremediation of dioxin by selecting the vital Edos for the mineralization of dioxin compounds.

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1. Introduction

Industrial activity over the past century has resulted in the release of many dioxins compounds in the environment which are formed as contaminating by products during the manufacturing of pesticides, herbicides and incineration of industrial wastes. These compounds cause cancer, also cause significant public concern because they are ubiquitously present in the food and serious affects on the immune system **(Kalantzi *et al.*, 2001).** Dibenzofuran and dibenzo-*p*-dioxins are used as model compounds of the dioxin (**Nojiri & Omori, 2002).** For biological technology to counteract environmental dioxin pollution, *Sphingomonas wittichii* RW1 is the unique strain capable to mineralize dioxin. The complete genome of RW1 was sequenced and a total genome size is 5,915,246-bp, consists of a main chromosome (5,382,261 bp) and two megaplasmids, designated pSWIT01 (310,228 bp) and pSWIT02 (222,757 bp), this nucleotide sequence is available under gene GenBank accession number CP000699 to CP000701 **(Miller *et al.*, 2010)**. The complete genome sequences of RW1 provide the possibility to detect the precise locations of the concerning genes sequences. Extradiol dioxygenases (edos) play a pivotal role in numerous degradative pathways, including the degradation of dibenzo-*p*-dioxin and dibenzofuran by *Sphingomonas wittichii* RW1, where such enzymes are responsible for the *meta* cleavage of the pathway intermediates 2,2´,3-trihydroxybiphenylether (THBE) and 2,2´,3-trihydroxybiphenyl (THB) **(D'Enza, 2002**). On the basis of sequence alignments, the extradiol group of dioxygenases has been divided into three families **(Vaillancourt *et al.*,2003),**type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily **(Gerlt & Babbitt, 2001),** the type II extradiol dioxygenases include enzymes such as protocatechuate 4,5-dioxygenase (LigAB) from *Pseudomonas paucimobilis* **(Noda *et al.*,1990),** which has two different types of subunits, the type III extradiol dioxygenases, including 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides sp*. strain KP7 **(Iwabuchi & Harayama,1998),**belong to the cupin superfamily **(Dunwell *et al.*, 2001).**The type I of extradiol dioxygenases (edos) was the aim of this study which usually involved in most aromatic compounds degradation, this type was annotated with the superfamily name as Glyoxalase/bleomycin resistance protein/dioxygenase on database. 2,3-Dihydroxybiphenyl has been shown to be a substrate for many extradiol dioxygenases, including DbfB (*edo1*), *edo2* , *edo3*, *edo4*.*edo5*, *edo6*, *edo7* and *edo8* from *Sphingomonas wittichii* RW1, (**Happe *et al.*, 1993;D'Enza, 2002; Aly, 2007)**. The *meta* cleavage reaction product is yellow colored providing an easy colorimetric test for a rapid screening of bacterial colonies carrying 2,3-dihydroxybiphenyl 1,2-dioxygenase activity. The aim of present study was to identify, characterize, clone, and expression of new

edos genes type I from RW1 genome, for contributing in dissolve the problem of degradation of dioxin compounds.

2. Materials and Methods

Growth of *Sphingomonas wittichii*

Sphingomonas wittichii RW1(No. 6014; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig; Germany) were cultured in 10 ml M9 phosphate- buffered minimal media **(Sambrook *et al.*, 1989)** at 30°C in a water bath shaker supplemented with dibenzofuran (DBF) crystals used as a carbon and energy source.

DNA extraction

Genomic DNA of *Sphingomonas wittichii* RW1 was isolated from cells pregrown on DBF according to the protocol for bacterial DNA extraction GeneJET™ Genomic DNA Purification kit (Fermentas Co).

Design primer for Edos genes from RW1

Three different specific primers sets (Fedo9RW1/Redo9RW1, Fedo10RW1/Redo10RW1, Fedo11RW1/Redo11RW1) were designed for the specific amplification of the three genes *edo9*, *edo10* and *edo11* of strain RW1 by using the corresponding CDS as input in the online program Primer3, available at: http://frodo.wi.mit.edu/. The three primers are indicated in (Table 1).

Table 1. The primers and its sequences used for the amplification of the three Edos (*edo9, edo10, edo11*) from *Sphingomonas wittichii* RW1

Primer	Sequence
Edo9F RW1	5` GGAGCCCAGCATGGCCATCAT 3`
Edo9R RW1	5` CGACAGGCGCCTAGATATCGGG 3`
Edo10F RW1	5` GCTGACGCCCGACCAGAGAGAT 3`
Edo10R RW1	5` GATCGGCCACGGCGGACAGG 3`
Edo11F RW1	5` GTGCGGCTGGTCAGCGCTGG 3`
Edo11R RW1	5` CTCGTATTAATATATGGAGC 3`

PCR reactions and conditions

PCR amplifications were usually performed on model T Personal thermocycler (Biometra), in a final volume of 50 µl, containing 0.5 µM of each primer, 0.2 mM of an equimolar dNTPs mix, 5µl of 10X PCR buffer, 2.5U of Taq DNA polymerase , as a template 1µl of genomic DNA with a concentration between 0.1 to 10 ng , the mix put in 0.2ml sterilized PCR tube. PCR conditions comprised an initial denaturation temperatures (94°C, 5 min) followed by 35 cycles of denaturation (94°C, 45 sec), annealing temperature (the optimal temp for primer) (50°C, 1 min) elongation (72°C, 2min), and a final elongation for 7 min at 72°C.This conditions used with three primers.

Gel electrophoresis

The PCR products (3 µl of the PCR reaction) were checked for integrity by agarose gel electrophoresis (1% agarose, 1/2X TBE buffer), PCR product was visualized by soaking in a dilute solution of ethidium bromide and used Gel Documentation System model 286-760 DigiDoc-It™ for the imaging and documentation of agarose gels.

Cloning of the three edos genes fragments

The amplified edos genes (*edo9*, *edo10*, and *edo11*) were ligated into pTz57R/T Vector (Fermentas Co) was carried out in a total volume of 15µl, comprising 1.5µl Vector (55 ng/µl), 3 µl of 5x ligation buffer, 1.5 µl PCR product, 0 .5 µl T4DNA ligase(5 u/µl, Fermentas) and 8.5 µl water nuclease – free. The ligation reaction mixture was incubated over night at

4°C. *E.coli* JM107 was used for preparation of competent cells using TransformAid Bacterial Transformation Kit and its protocol (fermentas). 2.5 µl of ligation mixture were added into new microcentrifuge tubes, and chilled on ice for 2 min, 50 µl of the prepared cells were added to each tube containing the ligation mixtured, mixed, incubated on ice for 5 min and plated immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plates. The plates were incubated overnight at 37°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel were used for determining the molecular weight of denaturated protein subunits, to check the efficiency of the protein purifications, and to show overexpression of the three genes separately cloned in pTz57R/T vector and were prepared as follows:

Separating gel

Acrylamide concentration	12,5%
crylamid/Bis (30%)	4.2 ml
4X lower Buffer pH 8.8 (182 g Tris base, 40 ml SDS (10%9 and 14 ml HCl (conc.) per liter)	2.54 ml
H_2O	2.34 ml
Glycerin	0.86 ml
TEMED	22 µl
APS 10 %	16 µl

Stacking gel

Acrylamide concentration	4%
Acrylamid/Bis (30 %)	0.533 ml
4X Upper Buffer pH 6.8 (61 g Tris Base, 40 ml SDS (10%) and 30 ml HCl (conc.) per liter)	1.0 ml
H_2O	2.467 ml
TEMED	10 µl
APS 10%	16 µl

The proteins of the samples were prepared from cell extracts of *E.coli* JM107 harbor vector with the insert from (*Edo9*, *Edo10* and *Edo11*) and the control was *E.coli* JM107 with vector without insert. All preparation were performed aerobically grown in 10 ml liquid media supplemented with IPTG, and ampicillin and incubated overnight. 2 ml from each sample harbor *edo 9*, *edo10*, *edo11* and control were centrifuged for 10 min at 10000 rpm, discarded the supernatant, 200 µl of extraction buffer were added for each sample and was applied for sonications for 1 min at 40 pulses with a Sonicator, the samples were centrifuged for 10 min at 10000 rpm, 10 µl of 2x treatment buffer were added to 20 µl from supernatant protein solution, incubated 95°C for 5min in a water bath, and loaded 30 µl of the samples into each well. The protein gels were stained overnight using a Coomassie brilliant blue stain and then were distained initially in an aqueous solution containing 30% methanol 10% acetic acid. PageRuler ™ Unstained Broad range Protein Ladder was used (Fermentas Co).

3. Results

PCR amplification of the three genes

For isolation of the three ORFs *edo9*, *edo10* and *edo11* from RW1 genome, three specific primers sets (Fedo9RW1/Redo9RW1,Fedo10RW1/Redo10RW1, Fedo11RW1/Redo11RW1) were designed. The results showed that amplification of 1004bp ,1151bp and 995bp fragments, respectively from genomic DNA of RW1 comprising the complete edo9, edo10 and edo11 ORFs, respectively (Fig.1 lane 1, 2, 3).

Expression of the three EDOs genes in *E.coli* JM107

The analyze of the edo9, edo10 and edo11 encode functional extradiol dioxygenase amplified fragments were separately cloned into pTz57R/T Vector. *E.coli* JM107 harboring only vectors without insert was applied as control. Screening of colonies carrying *edo9*, *edo10* and *edo11* inserts by spraying with 2,3 dihydroxybiphenyl respectively, colonies turned yellow that all exhibited extradiol dioxygenase activity, and thus that all three genes encode functional EDOs proteins.

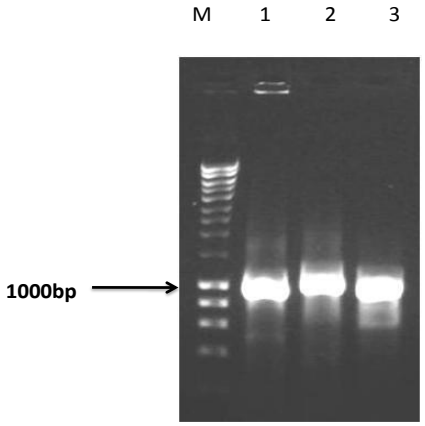

Fig. 1. Agarose gel electrophoresis of the 1004bp product obtained by using FEdo9/ REdo9 primer (lane1), 1151bp product obtained by using FEdo10/ REdo10 primer (lane2) and 995 product obtained by using FEdo11/ REdo11 primer (lane3), M, molecular weight marker hyperladder I (Bioline)

Analysis of the three Edos genes by SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the overexpression of *edo9*, *edo10* and *edo11* which were separately cloned on pTz57R/T were expressed and translated to polypeptides with the predicted sizes. The results explained the expression of *edo9* genes was observed at approximately ~ 40KDa (Fig 2A; lane1), *edo10* was observed approximately ~ 35KDa (Fig 2 B; lane 2`), and *edo11* was observed at approximately ~ 36KDa (Fig 2C; lane 1``**).**

4. Discussion

Over the past 2 decades, studies of the bacterium *Sphingomonas wittichii* RW1 have provided a wealth of knowledge about how this bacteria mineralize toxic dioxin pollutants dibenzo- *p* –dioxin (**Wittich *et al*., 1992; Wilkes *et al*., 1996**), and to co-metabolize a large number of chlorinated congeners of both dibenzo- *p* -dioxin and dibenzofuran (**Wilkes *et al*., 1996; Hong *et al*., 2002; Halden *et al*., 2005)**. These metabolic capabilities of *S. wittichii* RW1 are rare and potentially beneficial.

Extradiol dioxygenases play a key role in the metabolism of dibenzofuran, dibenzo-*p*-dioxin, PCBs and various other aromatic compounds. These enzymes utilize non-heme ferrous iron to cleave the aromatic nucleus *meta* (adjacent) to the hydroxyl substituents, incorporating both atoms of dioxygen into the product. The ferrous iron of these enzymes is coordinated by two histidines and one glutamate **(Han *et al*., 1995)** in which has been termed the 2-His-1-carboxylate facial.

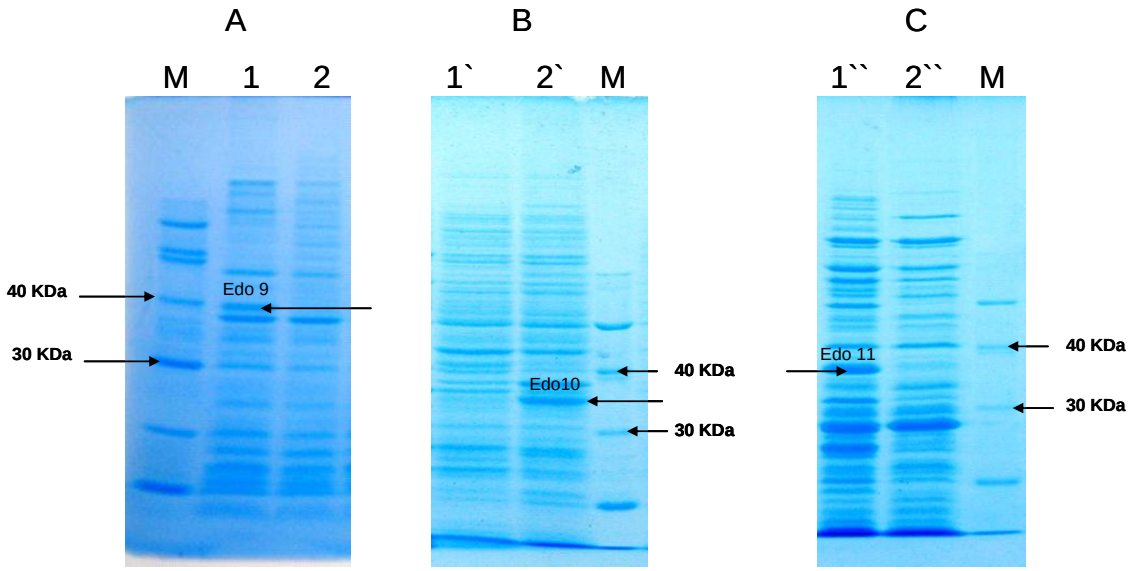

Fig. 2. SDS-PAGE analysis of cell extracts of *E.coli* JM107 with Edo9 insert (A) lane 1(40kDa), and its control lane2; Edo10 insert (B) lane 2``(35kDa), and its control Lane1`; Edo11 insert (C) lane 1`` (36KDa) and its control Lane 2``, and M Page Ruler unstained protein ladder.

In the current investigation we could show, that RW1 contains at least eleven extradiol dioxygenases type I as indicated in phylogenetic tree (Fig 3), which all exhibit activity against 2,3-dihydroxybiphenyl. The existence in some bacteria of multiple extradiol dioxygenases is well established **(Asturias & Timmis, 1993; Kosono *et al.*, 1997; Schmid *et al.*, 1997; Taguchi *et al.*, 2004)** and may indicate the capability of these strains to metabolize various aromatic compounds **(Taguchi *et al.*, 2004).** However, in the case of *S. wittichii* extradiol dioxygenases, the actual function of these genes can only in a few cases be deduced from the genetic environment. In the present study, three distinct *meta*-cleavage dioxygenases, *Edo9*, *Edo10*, and *Edo11* were genetically and biochemically characterized. Edo9, Edo 10, and Edo 11 have conserved for 3 domains first domain TIGR03213 (2,3-dihydroxybiphenyl 1,2-dioxygenase), second domain cd08360 (C terminal domain of *Burkholderia* sp. NF100 MhqB and similar proteins; MhqB is a type I extradiol dioxygenase involved in the catabolism of methylhydroquinone, an intermediate in the degradation of fenitrothion), and third domain cl14632 (This domain superfamily is found in a variety of structurally related metalloproteins, including the type I extradiol dioxygenases, glyoxalase I and a group of antibiotic resistance proteins). As mentioned before, *edo9* (Swit_2311) has a 48% similarity with *edo4* **(Aly, 2007)** and previous by a gene, the product of which exhibits about 40% of sequence identity with vanillate monooxygenase. presence vacillate monoxygenase before edo9 (indicate that this protein for edo9 enzyme involved in the catabolism of methylhydroquinone, an intermediate in the degradation of fenitrothion **(lim *et al.*, 2012)** i.e related to the second domain type, edo 10 (Swit_1680) has followed by Aromatic-ring hydroxylase-like FAD Binding monooxygenase this monooxygenases incorporate one hydroxyl group into substrates and are found in many metabolic pathways. In this reaction, two atoms of dioxygen are reduced to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NAD(P)H **(Harayama *et al.*,1992)**. P-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* contains this sequence motif (present in in flavoprotein hydroxylases) with a putative dual function in FAD and NADPH binding **(Eppink *et al.*, 1999)**, while edo 11(Swit_1564)) has a 47% similarity with *edo2*, which was briefly described **(Armengaud *et al.*, 1998),** and encodes a protein with only low sequence similarity to any other known extradiol dioxygenase, sharing 43% amino acid sequence identity with 2,3-dihydroxybiphenyl 1,2-dioxygenase (BpdE) from *Rhodococcus* sp. M5 **(Wang *et al.*, 1995)** and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC3) from *Rhodococcus* erythropolis **(Kosono *et al.*, 1997)**. All the three edos were cloned and showed as function genes transformed 2,3-dihydroxybiphenyl to the yellow *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, this means that all the three edos belong to the first domain type I 2,3-

dihydroxybiphenyl 1,2-dioxygenase, the third enzyme of a pathway for biphenyl degradation, and act on a range of related substrates.

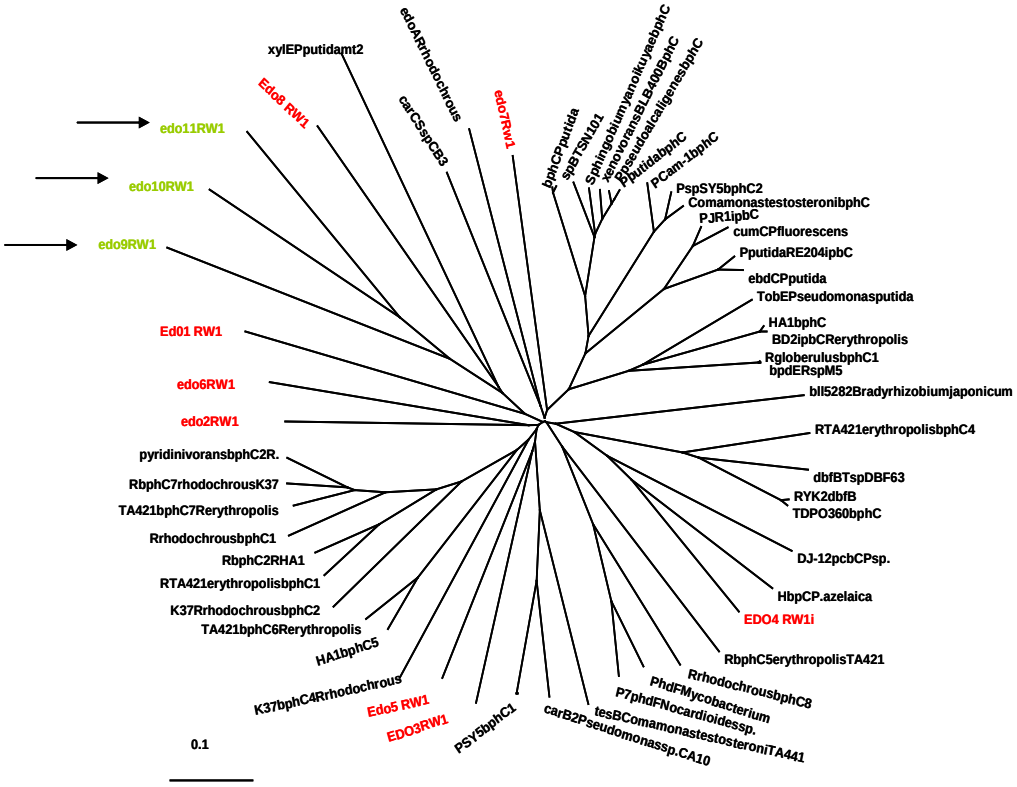

Fig. 3. Phylogenetic tree shows the eleven extradiol dioxygenases typeI from *Sphingomonas wittichii* RW1 and the three arrowd points to the three new extradiol dioxygenases described in this study (Edo 9, Edo10, and Edo11).

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