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EXPRESSION OF THE *CAENORHABDITIS ELEGANS* ARYL HYDROCARBON RECEPTOR LIGAND BINDING DOMAIN

By

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A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Biomedical Science

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To my Parents, Wife, Doaa, and daughters, Mariam and Razan…

Declaration

The work presented in this thesis is my own unless otherwise stated. Information from other sources has been fully acknowledged. No part of this thesis has been previously submitted for examination leading to the award of a degree.

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ABSTRACT

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, which mediates the potent toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. AhR is regulated by the ligand-binding domain (LBD) of the AhR, and so determining how the binding of ligand activates AhR is of considerable interest. However, there are no structural data on mammalian AhR LBDs, and expression of the mouse AhR LBD in *E. coli* yields insoluble protein. Expression in more complex systems, such as insect cells (*Spodoptera frugiperda*), yields soluble AhR LBD, but only ~10% of the total protein is in a ligand-binding competent form.

In order to address the structure of the AhR LBD, we have used a model system. There is good amino acid sequence similarity between human AhR and *C. elegans* AhR (CeAhR). We have investigated whether the three dimensional structure of CeAhR LBD will help in understanding this structure in mammals.

CeAhR LBD was cloned into the vector pRSET to give histidine-tagged protein. The clones were then transformed into *E. coli* BL21(DE3) or Arctic Express strains, followed by induction with IPTG. Bacteria were lysed and 100000g supernatants were prepared. Proteins were purified by Ni^{2+} affinity chromatography.

Expression of recombinant proteins in the bacterial system revealed that the induced protein from the pRSET.CeAhR LBD construct was ~29 kDa, as predicted. Large amounts of these proteins were produced (~5-10% of total

bacterial protein) and the vast majority was insoluble. However, on preparation of a 100000g supernatant, the samples yielded small amounts of soluble CeAhR LBD fusion protein. This is in contrast to results obtained with mouse AhR LBD, which yielded no detectable protein in a 100000g supernatant. The CeAhR LBD proteins were successfully purified by affinity chromatography and were obtained in good yield from the original cytosols. However, the yield of soluble AhR fusion protein was ~100 microgrammes of protein per litre of BL21(DE3) bacterial culture. The experiment was repeated using Arctic Express bacteria, which have a constitutively expressed chaperonin, and express at 12°C. However, the yield of protein was similar, at ~100 microgrammes of protein per litre.

Thus the CeAhR LBD yields soluble protein in a bacterial expression system, but the levels of expression are too low to enable this protein to be purified for use in structural studies. Trials to express CeAhR LBD in transgenic *C. elegans* and *Pichia pastoris* yielded no soluble protein.

The research moved to look for ligands for CeAhR by using a lethality test with *C. elegans* in vivo studies. The results showed that TCDD and AZ1c (from AstraZeneca) affect the wild type *C. elegans*, but without killing them. Repeating this test on AhR null animals showed that the effects were abolished. Thus the CeAhR is a receptor that appears to bind TCDD and AZ1c, albeit weakly, contrary to previous reports in the literature.

ACKNOWLEDGEMENTS

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1 INTRODUCTION

1.1 2,3,7,8-tetrachlorodibenzo-p-dioxin and its toxic effects

2,3,7,8-tetrachlorodibenzo-p-dioxin (Collier et al., 2008) (Figure 1) is a known toxin that induces a wide range of toxic effects including hepatic toxicity, teratogenic disorders, and reproductive and carcinogenic effects (Pocar et al., 2005; Schwarz and Appel 2005) TCDD is also responsible for neurological and immunological manifestations (Dzeletovic et al., 1997). TCDD is often referred to as simply “dioxin”, although, strictly speaking dioxin is only a part of this molecule (Figure 1). It is considered one of the most toxic planar halogenated hydrocarbons, and is a waste product of industrial processing of organic compounds containing halogens. Another major source is chlorine bleaching of paper pulp. Burning wood produces TCDD and related dioxins that are stable in the ecosystem (Lohmann et al., 2006; Pandelova et al., 2009). These compounds accumulate in the food chain in the fatty compartments of fish, birds and mammals and dioxin particles can stick to the top of plants (Clarkson 1995; Pohl et al., 1995; Loonen et al., 1996; Meulenbelt and de Vries 2005; Kamphues and Schulz 2006). They are lipophilic compounds that stay in the body for many years. The half life of TCDD in humans is at least 7 years (Birnbaum 1994). There are 75 different compounds, which are considered to be polychlorinated dibenzo-p-dioxin family members. These compounds differ in the number and location of the chlorine atoms (Figure 1). The most dangerous structures are the tetra-chlorinated ones,

because they are very difficult to metabolise, and this is why it has a very long half life in the body (Leung et al., 2006).

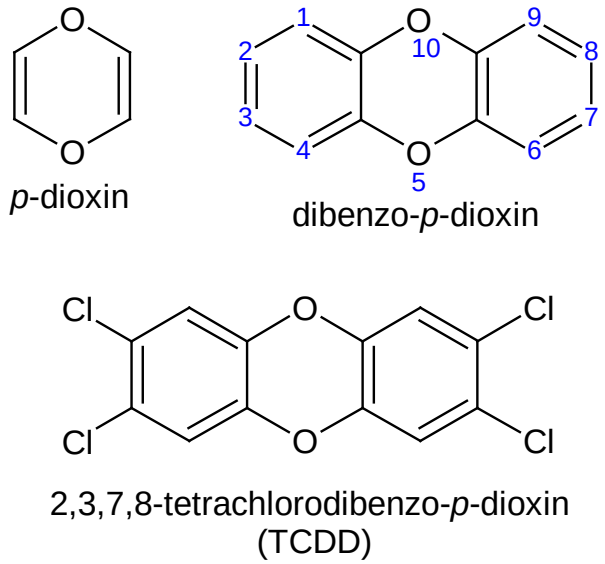


Figure 1. The chemical structures of *p*-dioxin, dibenzo-*p*-dioxin with the ring numbering system and TCDD.

The toxicity of TCDD became the focus of attention since the early 1970s, when 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) derivatives were used as herbicides in the USA and Vietnam. During the war in Vietnam, Agent Orange, a 1:1 mixture of the n-butyl esters of 2,4-dichlorophenoxyaceticacid (2,4-D) and 2,4,5-T, was sprayed over a wide area as a chemical weapon (Kramarova et al., 1998; Schecter et al., 1998; LaKind and Filser 1999). The toxic effects were detected in pregnant mothers and the biological manifestations were confirmed in rodents. The teratogenic effect of 2,4,5-T was due to the presence of TCDD as part of the

mixture. For 40 years, research has been ongoing on dioxin and its molecular mechanisms. The toxic effects of dioxin depend on many factors, including the dose, the route, the species, and the age and sex of the animal. For example, guinea pigs are very sensitive while the hamster is relatively resistant. The half-life of dioxin in rodents is just weeks, in comparison to years in humans due to the fatty component inside the body. The toxic effects range from nausea, vomiting, headache, chloracne, psychological problems and various cancers. TCDD is a well-known non-genotoxic carcinogen (Dencker 1985; Cole et al., 2003; Abalos et al., 2010). It is also said that dioxin promotes cancer progression in humans and animals. Experimental chronic exposure to TCDD induces chronic wasting syndrome and is responsible for the atrophy of the lymphoid tissue, including the thymus. TCDD also has immunotoxic effects, as it affects the maturation of T cells, reduces cell proliferation and reduces cytokine secretions, and this is claimed to reduce the anti-tumour immunity. TCDD contamination is responsible for hepatoxicity, thyroid dysfunctions and the development of Diabetes Mellitus (Pearce and Braverman 2009; Chang et al., 2010). It affects gonadal maturation in males and induces placental dysfunction (Dencker 1985; Cole et al., 2003; Abalos et al., 2010) and miscarriage in females (Sharara et al., 1998). TCDD induces skin lesions and carcinogenic effects (Lin et al., 2003). Acute TCDD toxicity induces wasting and weight loss in experimental animals. TCDD suppressed the expression of genes that affect lipid and glucose metabolism, like *Hmgcr*, *Fasn*, *Srebf1* which are involved in diabetes (Sato et al., 2008).

1.2 AhR identification

TCDD promotes its toxic effects through activation of a protein receptor known as the aryl hydrocarbon receptor (Hankinson 1995; Ko *et al.*, 1996; Sato *et al.*, 2008). It has been shown that intracellular calcium control interacts with the regulation of target genes affected by AhR stimulation (Monteiro et al., 2008).

AhR is responsible for regulation of multiple genes, which leads to immunologic, hepatic and skin toxicity. It is noteworthy that transgenic mice over-expressing AhR develop spontaneous cancer (Tauchi et al., 2005). Related research showed that down-regulation of the aryl hydrocarbon receptor repressor (AhRR) was responsible for increased incidence of cancer in colon, breast, lung, stomach and cervix. AhR and AhRR work in a feedback loop to regulate each other. AhR activation stimulates the expression of AhRR, and at the same time AhRR down regulates the expression of AhR (Zudaire et al., 2008). Thus, the implication is that a decrease in AhRR leads to carcinogenesis through greater constitutive activity of AhR.

AhR may contribute to physiological functions, for example, cell proliferation, differentiation, motility and migration (Kleman et al., 1994; Seidel et al., 2000).

The creation of AhR-knockout mice enabled a focus on the role of AhR in normal growth. The results showed that AhR null mice exhibit reduced liver weight and portal fibrosis (Schmidt et al., 1996). Recent research work showed that AhR null mice express high levels of TGF-β1 and TGF-β3 factors (Transforming Growth

Factor Beta) that are expected to induce liver fibrosis and liver cell apoptosis (Chang et al., 2007). So it is proposed that AhR has triple functional activity. The first function is the adaptive metabolic response to PAH (Polycyclic Aromatic Hydrocarbons), the second effect is the toxic response to TCDD and the third is the developmental role in liver and vascular growth. The former two effects are mediated by known ligands, but the last one is not clear. In order to understand how AhR regulates liver growth and vascular development, experiments have been performed to create a hypomorphic allele of the AhR locus and dioxin was given to the growing embryo. The results showed that dioxin protected the growing mice from the liver and the vascular malformations seen in AhR hypomorphic mice. This experiment suggests that an endogenous ligand may be required for normal AhR function (Walisser et al., 2005).

Differences in the susceptibility to dioxins in the same species and even the same strain raised the question of the genetic polymorphism before the AhR was discovered. PAH induced CYP1A1 (final target of AhR stimulation) in mice liver. Crossing and back crossing of multiple inbred mice strains resulted in the identification of the AhR genetic locus that is responsible for induction of CYP1A1 before the discovery of AhR receptor protein. Adverse mutation of that locus blocks the response of CYP1A1 to PAH related compounds. A soluble cytosolic receptor model for AhR was suggested to promote aryl hydrocarbon responses. Studies discovered 2 complexes, one about 6S and the other was about 9S in the rat liver extract. They bind radioactive TCDD but no signal was detected

from the extracts taken from mutated AhR locus (DBA/N2) mice. Furthermore, these complexes are protein in nature as they were affected by proteases but not affected by DNAse or RNAse. Competitive displacement of TCDD was achieved by other CYP1A1 inducers. Neither Phenobarbital (CYP inducer) nor steroid hormones managed to displace radioactive TCDD. A new step towards identification of AhR was the discovery of 2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin. This is a photo activatable ligand that binds covalently with the proposed AhR on exposure to ultraviolet light. This ligand helped in discovering 2 protein masses in Hepa-1 cells of 70 kDa and 95 kDa, although the first protein was just an artefact proteolytic fragment (Beischlag et al., 2008). In 1988, Perdew and Poland successfully purified AhR partially and later on antibodies against AhR were developed (Bradfield and Poland 1988). Co immune-precipitation of AhR resulted in discovery of Hsp90 as a chaperone protein. Primers of the human AhR locus were constructed and the AhR was cloned from human genomic DNA for further extensive characterisation in the 1990s by Oliver Hankinson (Hankinson 1995). This revealed proteins in the molecular weight range from 95 to 105 kDa and containing approximately 805 amino acids.

1.3 AhR structural domains

The AhR has four main structural domains (Figure 2): 1- basic helix-loop-helix (bHLH) domain; 2- PAS-A domain; 3- PAS-B domain; 4- glutamine-rich domain.

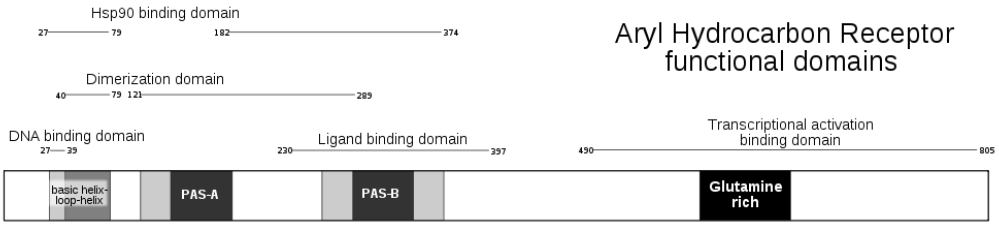


Figure 2. Diagram of the various domains of the mouse AhR adapted from (Fukunaga et al., 1995) .

Studies were carried out to analyse the two PAS (PER: circadian clock protein, ARNT: aryl hydrocarbon receptor nuclear translocator protein, SIM: single minded protein) domains of AhR. The PAS-A domain is involved in the interaction between AhR and other proteins. Removal of PAS A causes weak dimerisation with ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) and stops transcription. Removal of PAS-B produces an AhR that is capable of binding ARNT, but not activating ligands. AhR PAS B is supposed to bind ligands and chaperones, including Hsp90 (Heat Shock Protein), p23 (co-chaperone of the AhR complex) and AIP (Aryl hydrocarbon receptor Interacting Protein) (Denison et al., 2002). The pocket that acquires ligands, i.e. the Ligand Binding Domain (LBD), is included partially in the PAS B domain (Figure 2) (McIntosh et al., 2010). Therefore, in AhR the PAS B domain has wider interactions than PAS A. The bHLH is mainly related to binding DNA and Hsp90. The transactivation domain is dedicated to binding the dioxin response element (DRE), the binding site on DNA that promotes gene expression. The DRE has conservative DNA structure in various species (Sun et al., 2004).

1.4 PAS domain proteins

PAS domain proteins are a group of conservative 3-dimensional structure proteins, despite their heterogeneous primary structure. They are widespread in the animal kingdom from bacteria to humans. All these proteins contain alpha helices and beta sheets. The protein structure contains a HLH (helix-loop-helix) motif at the N terminal site followed by the PAS domain (Card et al., 2005; Pandini and Bonati 2005; Imamoto and Kataoka 2007). Experimental studies cut the HLH domain, and the PAS domain is still able to fold properly in the case of the PAS domain of PYP (photo active yellow) protein. This result is very important, as it is claimed that the AhR LBD could fold properly without expressing the full length AhR. Computer simulation studies propose that the PAS domain proteins have a common 3-dimensional structure. Furthermore, the simulations are optimistic in that they indicate that these proteins bind ligands in the same way (Chapman-Smith et al., 2004). The discovery of the crystal structure of any member of these proteins would be a major step forward, allowing us to know more about the structure of the whole group, that is to say one PAS domain protein is a good template for other members of the group. The first member of these protein domains was discovered in the Drosophila clock protein, PER (Period Protein). The second was the ARNT protein. The majority of PAS domain proteins work as environmental sensors (Figure 4), for example, PYP, FixL (The Oxygen Sensor

Protein), HERG (potassium channel protein), LOV (Light Oxygen Voltage protein), ARNT and AhR (Vreede et al., 2003).

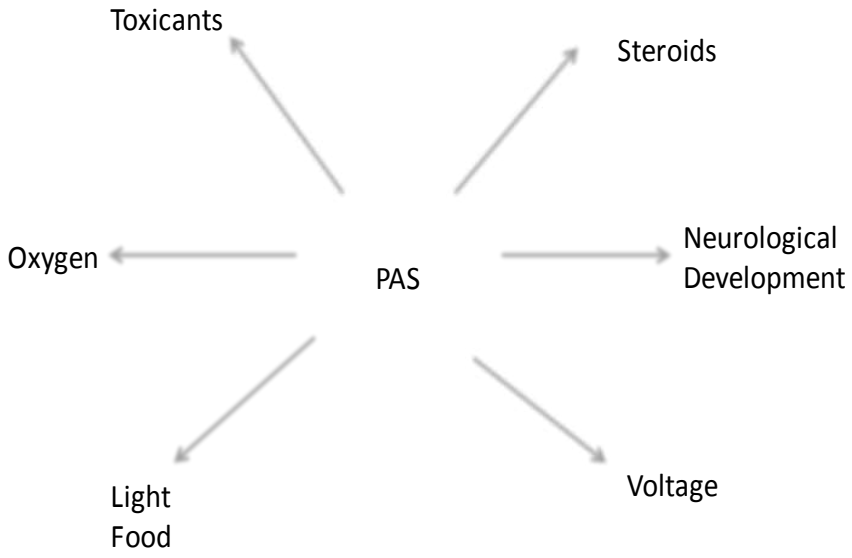


Figure 3. Diagram representing the functions of PAS domain proteins as environmental sensors (McIntosh et al., 2010). PAS domain proteins can act as light sensors like PYP, oxygen and energy sensors, voltage sensors of a potassium channel , neuronal regulators like C. elegans AhR, toxin metabolism like mammalian AhR and as part of a steroid receptor.

Currently, 34 mammalian proteins are considered to be PAS proteins. Furthermore, thousands of PAS proteins were discovered in many other species ranging from bacteria and plants to humans. The discovery of this huge number of proteins was attributed to the use of expanding data in the Genebank.

Mammalian PAS domain proteins play an important role in the hypoxia response pathway. During periods of low oxygen, the hypoxia inducing factor (HIF alpha) is released to help anaerobic respiration, angiogenesis and an increase in red blood

cell production. At normal levels, oxygen helps proteolysis of the normally produced HIF. With increase in hypoxia, the degradation of HIF decreases with net result of increased PAS domain containing HIF. The protein moves to the nucleus and binds ARNT, similar to AhR, and finally stimulates DNA to produce different enzymes needed for the target function (Kamae et al., 2010; Pasanen et al., 2010; Wenger and Hoogewijs 2010).

The second important PAS domain function is the dioxin response pathway. The ligand binds to the PAS domain protein, AhR, where it translocates to the nucleus and binds to ARNT, and then the complex binds the DNA response element (see section 1.7 for more detail). It is a similar transduction pathway to that of HIF alpha.

The third function of mammalian PAS domain proteins is maintenance of the circadian rhythm of the animal. Most organisms have the ability to adapt with the diurnal changes of light and darkness. These changes are called the circadian rhythm. In humans, the sleep wake cycle is regulated by endogenous biological clocks that exist in every organ. However, the master biological clock rests on the optic chiasm as part of the supra-chiasmatic nucleus. These biological clocks control the different biological changes between day and night. For example, the diurnal changes in blood pressure, the immune function and pituitary secretion are changed according to the function of the biological clock (Qu et al., 2010). Many genes are regulated by the circadian rhythm, including the PAS domain repressor proteins. These proteins are translated and phosphorylated, then they translocate to

the nucleus and bind to ARNT. The complex interacts with the DNA response element expressing a battery of genes that promote sleep, metabolism and other physiological changes (Hennig et al., 2009; McIntosh et al., 2010).

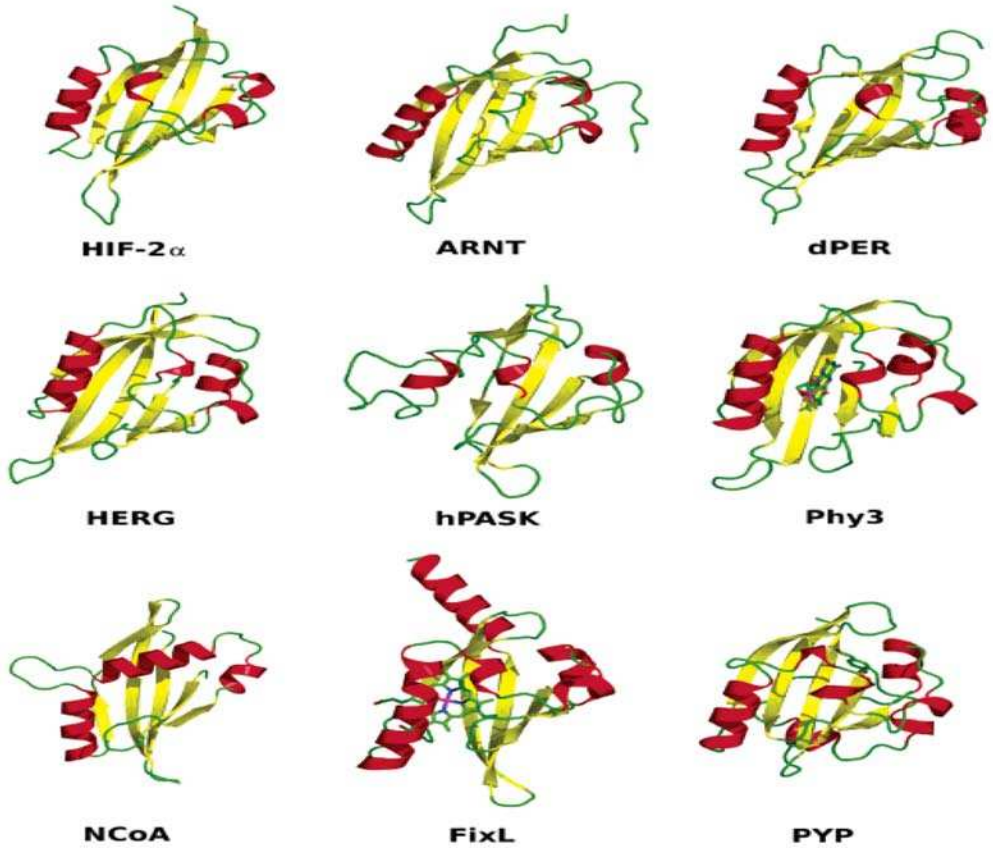


Figure 4. Different PAS domain structural models: HIF-2α, ARNT, dPER, HERG, hPASK, Phy3, NCoA, FixL and PYP. Secondary structure has been predicted by the Kabsch and Sander method. Both FixL and PYP models were created according to their X-ray structures (Pandini et al., 2007).

1.5 Endogenous AhR agonists

Some researchers have hypothesised that AhR is a receptor without a specific ligand, but there are several endogenous AhR agonists including bilirubin, biliverdin, metabolites of tryptophan, indirubin, indigo and other compounds. Typically, these compounds can activate AhR, but with less potency than TCDD. The functions of these, and other endogenous AhR ligands is unclear (Petersen et al., 2006). There are two theories; the first is that AhR has a physiological ligand that, as yet, is unidentified. It is supposed that the ligand, factor or hormone modulates the physiological AhR function, especially during embryonal development. AhR mutated mice change their phenotype without any xenobiotic exposure, supporting the theory that AhR has a physiological function. The battery of genes stimulated by the AhR-ARNT complex also indicates that AhR has a physiological function. The other theory claims that AhR is an orphan receptor that has an adaptive environmental function. It is thought that AhR detoxifies endogenous products during development. AhR can bind multiple endogenous compounds with different structure. This makes understanding the mechanism of ligand receptor binding interesting. These different endogenous chemicals are claimed to be waste products that are harmful to both embryo and adults. A third theory exists that is a mix of both mentioned models suggesting that AhR has dual function; one function is xenobiotic metabolism and the other function is supporting embryonic maturation and normal physiology.

The big classes of AhR agonists include the halogenated aromatic hydrocarbons, the family of polychlorinated biphenyls and the polycyclic aromatic hydrocarbons. On the other hand, there are various endogenous groups of chemicals, for example, indigoids, arachidonic acid metabolites and heme metabolites.

Indigoids are weak endogenous AhR ligands. Indirubin is supposed to be the most potent indigoid in binding AhR but is 100 times less potent than TCDD (Prochazkova et al., 2010). Some studies consider indigoids as AhR agonists, others consider them as partial agonists. These chemicals can be found in human urine below nanomolar level. The antagonistic effects of indigoids on transformation have been studied. In mouse hepatoma, expression of CYP1A1 is reduced in Hepa-1c1c7 cells loaded with indigoids, and indirubin in particular. The translocation of AhR into the nucleus was inhibited. On administering 10mg/kg body weight/day orally to mice for three consecutive days, the CYP1A1 expression in liver did not increase for indigoids (Nishiumi et al., 2008). Quinone reductase induction was recorded on supplementation with indirubin and indigo. These experiments concluded that indigoids are considered as antagonists of the AhR receptor, especially for low amounts in mice (Nishiumi et al., 2008).

1.5.1 Equilinin (3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one)

This weak endogenous ligand works as an AhR agonist. It is known to induce CYP1A1 and is considered an oestrogen derivative. It is used in hormonal replacement therapy for post-menopausal women (Bhavnani and Woolever 1981;

Orstan et al., 1986). In common with other AhR ligands, it has a planar structure that differs from other oestrogens (Jinno et al., 2006).

1.5.2 (1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)

ITE is an AhR agonist capable of stimulating the DNA response element. Gestational exposure of ITE did not show teratogenic manifestation. Some studies claim that ITE is a physiological ligand of AhR, however, there is no evidence that ITE is present in humans. ITE is known to be a potent murine AhR agonist. Experimental studies carried out on lung fibroblasts showed that ITE induces a wide variety of genes, similar to TCDD, but without manifest cytotoxicity. The difference between TCDD and ITE is attributed to the short half life of ITE in comparison to the persistent TCDD (Henry et al., 2010).

1.5.3 Arachidonic acid metabolites

Studies showed that there is a link between AhR transduction and prostaglandin synthesis. Lipoxin A_4, an arachidonic acid metabolite, is suggested to selectively bind AhR. Lipoxin A_4 both stimulates AhR (Schaldach et al., 1999; Sanchez et al., 2010) and inhibits (SOCS)-2 (suppressor of cytokine signalling 2) transduction, which is considered an anti-inflammatory action (Machado et al., 2006). This compound is an interesting focus of research, because its structure is unique. It was found to differ widely from TCDD in chemical structure and ligand binding, in structure studies performed to measure the mRNA of CYP1A1 on adding lipoxin A_4 to Hepa-1 cells. The results concluded that Lipoxin A_4 stimulated the AhR complex and induced CYP1A1 (Schaldach et al., 1999).

1.5.4 Heme metabolites

Heme metabolites are considered AhR agonists. Experimental studies on diseased rats with jaundice manifestation showed induction of CYP1A1. Heme, biliverdin and bilirubin, the most potent, are supposed to bind AhR (Sinal and Bend 1997; Phelan et al., 1998) and induce translocation to the nucleus, where the DNA response element is activated, inducing a battery of genes, including the mRNA of CYP1A1. In vitro experiments were carried out on the effect of hemin, biliverdin, and bilirubin on expression of CYP1A1 in Hepa-1c1c7 cells (Sinal and Bend 1997). A dose-response curve showed an increase in CYP1A1 induction by all three chemicals. The positive results appeared after 1 hour with biliverdin or bilirubin, however, the increase in CYP1A1 mRNA took more than 2 hours with hemin. Hepa-1c1c7 cells supplemented with these compounds induced a dose-response curve of CYP1A1-dependent 7-ethoxyresorufin-O-deethylase (EROD) activity with the best response generated by bilirubin (Sinal and Bend 1997).

1.5.5 Tryptophan metabolites

Tryptophan metabolites can bind AhR and promote transformation of the AhR complex. Furthermore, tryptophan and its metabolites induce CYP1A1 in rat liver cells. AhR is considered part of the protective metabolising system that protects from toxic metabolites, like tryptophan, that could be produced in excess by the normal microflora or due to contamination from the external enivironment (Schrenk et al., 1999).

1.5.6 Dietary compounds

Some dietary compounds can act as AhR agonists. Candidates include Indole-3-carbinol derivatives and natural flavinoids. The first group of compound can be found in vegetables like broccoli, and the second, especially plant polyphenols, can be found in fruits and vegetables (de Waard et al., 2008; Nguyen and Bradfield 2008).

1.6 Polycyclic aromatic hydrocarbons (PAHs)

Animal studies showed that exposure to some polycyclic aromatic hydrocarbons (PAHs) induced breast cancer, which is partly due to stimulation of AhR-mediated transcription, thereby inducing CYP family members that oxidise PAHs into carcinogenic intermediates (Trombino et al., 2000). Adding coal tar to rabbit's ear induces pre-cancerous papillomas that transform to cancer; this was recorded in the 1930s. PAHs were the active carcinogenic substances that exist in the coal tar (Filatova et al., 1973; Bickers and Kappas 1978; Lycheva et al., 1990; Liu et al., 2009). The PAHs are metabolised by CYP1A1 into more toxic intermediates that induce DNA damage (Mahadevan et al., 2007). On the other hand, PAHs are, like TCDD, AhR agonists that transform AhR into the nucleus and stimulate the DNA response element to produce a battery of proteins and enzymes, but less potently. At the same time, PAHs induce active metabolites that induce the CYP1A family and metabolise PAHs themselves in a cycle that takes from 12-24 hours. Studies carried out on human lymphocytes showed that cells treated with PAHs in vitro

were transformed into malignant lymphoplasts (Saurabh et al., 2010). Other studies looked at the relationship between PAHs and bronchogenic carcinoma. These effects were demonstrated in cigarette smoking, which is related to cancer of the lung, larynx and oral cavity. Cigarette smoke contains hundreds of chemicals, including PAHs (Nebert et al., 1993; Nebert et al., 2000; Nebert et al., 2004). In addition, burning organic material is an important source of PAHs; yet, the major source is oil spills. Unfortunately, these compounds are lipophilic and stay in the ecosystem for a long time. PAHs are also produced during cooking; for example, vegetables cooked multiple times at high temperature develop a group of carcinogenic compounds, including PAHs (Srivastava et al., 2010; Srivastava et al., 2010).

1.7 AhR signalling pathway

The AhR-receptor contains a basic helix-loop-helix (bHLH) DNA binding domain (Abel and Haarmann-Stemmann 2010) and two PAS domains, which are a sequence motif related to that in the Drosophila circadian rhythm regulatory protein, *period* (Dzeletovic et al., 1997). Once TCDD combines with AhR, it undergoes conformational changes and the AhR/chaperone complex translocates to the nucleus (Figure 6). The AhR then dissociates from its chaperone proteins (Hsp90, AIP, and p23) and following the association with another protein, ARNT, it binds to DNA and induces transcription (Figure 6). ARNT also belongs to the bHLH-PAS family (Mary et al., 2005). One of the chaperone proteins is Hsp90,

which dissociates from the TCDD/AhR complex after translocation to the nucleus. Inside the nucleus, the AhR-ligand-ARNT complex binds to a specific DNA motif called the dioxin response element (DRE) (Dzeletovic et al., 1997; Stevens et al., 2009).

AhR stimulates transcription of many genes encoding different metabolising enzymes, like the cytochrome P450 family, e.g. CYP1A1, glutathione transferase, UDP-glucuronyl transferase, NADPH and quinone oxidoreductase (Tang et al., 2008).

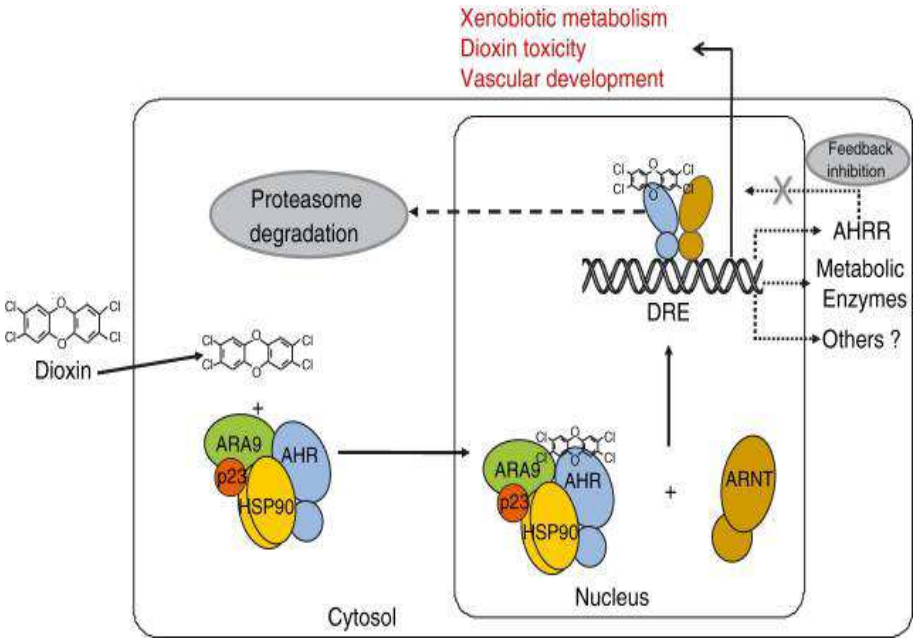


Figure 5. Diagram of the AhR pathway following binding with ligands like dioxin (Stevens et al., 2009). DRE is Dioxin Response Element. ARA9 is another name for the chaperone AIP. Hsp90 is heat shock protein. ARNT is the aryl hydrocarbon receptor nuclear translocator protein.

1.8 Composition of AhR complex and chaperones

Non-liganded AhR is found in the cytosol complexed with chaperone proteins including two Hsp90 molecules (a heat shock protein of 90 kDa weight), AIP (also known as ARA9 and XAP2) and p23.

Hsp90 keeps the receptor stable in the cytoplasm and prevents it from going to the nucleus. The function of p23 is not fully understood, but it is thought to support the interaction between AhR and Hsp90 (Stevens et al., 2009). AIP is supposed to help proper folding and signalling of AhR. On binding of the ligand, the AhR complex moves to enter the nucleus and Hsp90 and the other chaperones dissociate from the complex leaving ARNT to bind AhR prior to signalling (Stevens et al., 2009). Hsp90 is a tetrameric chaperone protein that maintains AhR in its proper configuration. Hsp90 is known to stabilise other receptors like the glucocorticoid receptor. Cancer cells produce Hsp90 in huge amounts to induce metastasis and thus are a target of anti-cancer research to arrest metastasis. Hsp90 is formed of 3 domains, where the N domain is the amino terminal domain, and the M domain is the middle domain that binds the AhR or other interacting protein, while the C domain is the carboxyl terminal domain that is a target for drugs or co-chaperones (Dao-Phan et al., 1997; Trepel et al., 2010). On the other hand, *C. elegans*' Hsp90 has a different function and does not stabilise or bind AhR (Powell-Coffman et al., 1998). Mammalian Hsp90 binds to AhR at two different sites, and by binding to the AhR complex it keeps its configuration in a state that is stable in the cytoplasm. Studies have been carried out to analyse the function of

Hsp90 in relation to AhR. Experimental loading with geliandamycin that inhibits Hsp90 binding resulted in decreased AhR in the cytoplasm, because of the increased degradation. This experiment indicates the essential role of Hsp90 in maintaining the AhR complex in the cytoplasm (Bell and Poland 2000). Once ligand binds to AhR, it is subjected to subsequent change in topology and translocates to the nucleus where it dissociates from the binding chaperones. A process called transformation ends in the expression of a battery of genes producing different AhR effects (Kazlauskas et al., 2001; Petrulis and Perdew 2002).

A study by was carried out where a conditional mouse model was created (Nukaya et al., 2010). The AIP locus of the model mouse hepatocytes was deleted to study the mechanisms of AIP in AhR signalling. The study discovered two functions of the 330 amino acid AIP protein in AhR transduction. (i) The normal AIP induction in hepatocytes is important to keep cytosolic AhR protein in a stable state in the mammalian liver. (ii) Expression of the AIP chaperone is important for ligand receptor complex transformation, and promoting the hepatic toxicity of dioxins. The genes expressed by AhR are affected by AIP expression in heterogenous response. The genes of CYP1B1 and AhRR are AIP-dependent for dioxin-induced toxicity, while both CYP1A1 and CYP1A2 are not AIP dependent. These results indicate that the mammalian AhR-responsive elements are more than one group that would need more extensive research to understand the genes responsible for the toxic effects of dioxin on the liver (Nukaya et al., 2010). AIP has multiple

interactions, and AhR is one factor that binds AIP. On the other hand, AIP interacts with phosphodiesterase-4a5, a protein, which is essential for the function of cAMP. Deregulation of phosphodiestrerase inhibitor and down-regulation of cAMP is responsible for isolated familial pituitary adenoma, which is inherited in an autosomal dominant pattern due to germline mutation of the area encoding for AIP. The AIP protein inhibits degradation of AhR, prolonging its half life in the cytoplasm. The AhR-AIP interaction keeps the configuration of the AhR complex away from interacting with other transcription factors in the absence of the AhR ligands (Leontiou et al., 2008; Pesatori et al., 2008).

P23 is a co-chaperone protein, which is a member of the receptor complex group. It is part of the AhR chaperone complex that binds the N-terminal of Hsp90 chaperone. Before ligand binding to AhR, the complex chaperones protect the receptor from transformation. Without p23, it is thought that it is difficult for Hsp90 to dissociate from the AhR receptor before it binds ARNT. Also, the ligand affinity of AhR decreases without the help of p23. P23 has functions other than as part of the AhR receptor complex. It binds Hsp90 as a co-chaperone for steroid receptors, which is important in ligand affinity to the glucocorticoid receptor. Other functions include prevention of protein aggregation and playing a role in telomerase activity. Knock-down p23 is lethal to animals because of the defective lung function due to dysfunction of the steroid receptor that is essential for lung maturation in utero (Kazlauskas et al., 1999; Flaveny et al., 2009).

1.9 AhR and carcinogenesis

AhR was discovered by Poland and his group more than 30 years ago (Poland et al., 1976). It mediates most or all effects of dioxin and related compounds (Poland et al., 1976; Brauze et al., 2006). These chemicals are considered carcinogenic, where AhR creates a model for cancer mechanisms, and has intrinsic effects on the cell cycle without binding exogenous ligands. It inhibits the cell cycle progression and induces cell cycle arrest, which contradicts its role in cancer precipitation (Gramatzki et al., 2009; Ma et al., 2009; Barhoover et al., 2010). On exposure to TCDD, a full agonist of human AhR, the ligand receptor complex transforms and translocates into the nucleus resulting in expression of a battery of genes that play a role in phase I and phase II metabolism. Many transcriptional factors are induced either directly or secondarily and these modulate the cell cycle. TCDD results in an increase in tyrosine kinase (Blankenship and Matsumura 1997; Backlund and Ingelman-Sundberg 2005) and stimulates the MAP kinase pathway. These proto-oncogenes are involved in human hepatoma (Yim et al., 2004; Borlak and Jenke 2008). AhR induces the c-myc gene that is related to breast cancer (Yang et al., 2005; Jensen et al., 2006). The outcome of studies was that AhR induces FOS and JUN families that are considered oncogenes, which may participate in cancer development. The constitutively active CA-AhR-transgenic in, for example, B6C3F1-mice, showed development of stomach cancer and liver tumours, despite a decrease in body weight and increased apoptosis. This model may explain how AhR induces cancer and inhibits the cell cycle at the same time (Moennikes et al.,

2004; Marlowe and Puga 2005). AhR is involved in cancer through two mechanisms depending on the class of AhR agonists. The first group is HAHs (Halogenated Aromatic Hydrocarbons), which include TCCD. These are considered non-genotoxic carcinogens, producing cancer without genotoxicity. In inducing cancer, the mechanism involves 3 stages; the first is intiation, the second is promotion, and the third is progression. AhR stimulated by TCDD helped promotion of cancer and expanded the already initiated clone to produce cancer (Ellinger-Ziegelbauer et al., 2009). On the other hand, PAHs are AhR ligands metabolised by CYP1A1 into more toxic intermediates that induce damage to the DNA, that is to say genotoxic carcinogens. In addition, this is a second mechanism to induce cancer by activation of AhR. Regarding the crosstalk between AhR and TGFв, AhR null animals showed an increase in the level of TGFв. This factor regulates development, cell migration and apoptosis (Gomez-Duran et al., 2009). In rat models, continuous ingestion of TCDD for 2 years resulted in cancer of the lung, liver, plate and nasal turbinates. It is calculated that 1 ng per kg body weight per day for 2 years is sufficient to cause risk of developing cancer (Kociba and Schwetz 1982). To explain species difference in sensitivity to cancer, the ligand binding domain polymorphism and the C terminal region of AhR are responsible for different responses to the same ligand. Induction of CYP1A1 and CYP1A2 is linked to cancer production (Qian et al., 2010). These enzymes are related to increased metabolism of oestrogens and increase free radical production, which is indeed genotoxic. These data may explain why female rats are more susceptible to

hepato-carcinoma than males or humans, where CYP1A1 induction is less than in female rats (Schwarz and Appel 2005). In humans, the risk of cancer is less than rodents, but is related to lung, gastro intestinal tract cancers, soft tissue sarcoma, breast carcinoma and non-Hodgkin lymphoma (Safe 2001).

1.10 Evolution of toxic AhR

PAS-domain containing proteins are found in various organisms like animals, plants, fungi, bacteria and archea (Crews 1998; Pellequer et al., 1998; Somers et al., 2000). However, AhR domains exist in metazoans, and are diverse in multiple phyla, and in different species from which extensive genetic and developmental research has been performed. AhRs are present in many animal species, which provides the potential to study AhR function and evolution in a broad scope of organisms from metazoa to humans. These AhR candidates have common structural and functional properties considering some distinct criteria. Moreover, these features are considered as an evolutionary maturation of the function of AhR from metazoa to the complex adaptive functions in higher animals or humans, and explain how these chemicals interact with their receptors mediating ligand function and inducing toxicity (Hahn 2002).

The cDNAs of AhR have been successfully cloned from the genomic DNA of many mammalian strains and extensive molecular and biochemical studies on AhR proteins have been carried out in these different species with special interest in

mouse and human AhR (Hankinson 1995; Schmidt and Bradfield 1996; Hahn 1998). In mouse strains, minor polymorphism in amino acid sequences induces major differences in receptor binding affinity and subsequent functional effects, which could explain why each species member differs in response to dioxin toxicity (Ema et al., 1994; Poland et al., 1994; Wong et al., 2001). AhRs from other mammalian species have not been as well characterised or biochemically studied, and may have similar physiological properties (Gasiewicz et al., 2008; Vuori et al., 2008). AhR cDNA sequences of the very sensitive guinea pig and the very resistant hamster have been studied (Dencker 1985; Olson 1986; Gassmann et al., 2010). However, it is difficult to understand how polymorphisms in the ligand binding domain and the C terminal can explain the observed thousand-fold difference in sensitivity to dioxin. The AhR size varies from 95 to 125 kDa in various vertebrates. Furthermore the amino acid sequence is different from one animal to another. On the other hand, the N terminus structure is more conservative. The TAD (the Trans Activation Domain) shows both inter and intra species variation that reflects variable response to dioxin toxicity. The LD_{50} of TCDD in guinea pig is 1µg/kg, yet the LD_{50} in hamster is 1mg/kg. The DBA2 strain of mouse is 14 times more resistant than the C57BL6 strain; this is explained by an amino acid polymorphism in the AhR ligand binding domain. In addition, Han-wistar rats are 1000 times more resistant than Long-Evans rats. This is explained by a point mutation in the TAD. The resistant hamster TAD is long with more glutamine amino acids than sensitive species. Both TADs of guinea pigs and

humans look similar. The C-terminus ends of human and mouse AhR share identity of 58% in contrast to the highly conservative N-terminus (Ramadoss and Perdew 2005). Another area of research on AhR diversity is the presence of AhR polymorphisms in humans that affect the expression of CYP1A1 and contribute to the susceptibility to lung cancer. Four single nucleotide polymorphisms (SNPs) and the subsequent changes in amino acid sequence of the human AhR protein in the C-terminal TAD (exon 10) have been identified (Kawajiri et al., 1995; Watanabe et al., 2001; Harper et al., 2002; Rowlands et al., 2010). It is not clear how these polymorphisms play a role in dioxin susceptibility, CYP1A1 expression, chloracne or cancer production (Kawajiri et al., 1995; Anttila et al., 2000; Smart and Daly 2000). Yet, a study carried out on proteins translated by human AhR alleles having more than one SNP in a combination of two or three SNPs resulted in decreased induction of CYP1A1 gene transcription (Wong et al., 2001). However, the clinical application of these results is yet to be established.

Some marine species, like mouse strains, are extremely sensitive to dioxins. Their AhR binds to ligands with higher affinities than other species, and unfortunately they are exposed to a high amount of dioxins and other AhR agonists. This raises the question about the high risk of environmental toxicity of dioxins in the seas.

1.10.1 Birds, reptiles and amphibians

Studies have been applied to avian AhRs, where chickens were subjected to dioxin intoxication, and extensive research undertaken and updated every day (Head and Kennedy 2010). Some birds living near water are in danger of intoxication from

environmentally-stable dioxin and its related compounds (Gilbertson et al., 1991). As mentioned previously, there is a wide range of levels of affinity to dioxins in different types of birds, indicating the importance of molecular research on AhR in these animals (Karchner et al., 2006; Pirard and De Pauw 2006; Head and Kennedy 2010). Biochemical research on chicken AhR showed that they are sensitive to dioxin toxicity due to high TCDD binding affinity (Sawyer et al., 1986; Karchner et al., 2006). Biochemical characterisation of AhR in birds is still primitive. The full length sequences of AhR are available only in 2 species, chicken and common tern (Karchner et al., 2000; Head and Kennedy 2010). Even less research data is available about AhRs in amphibians and reptiles. AhRs have been identified in newt (Marty et al., 1989) and a turtle (Hahn et al., 1994). The cDNA of AhR has been cloned from mudpuppy (Karchner et al., 2000) and a frog (Collier et al., 2008). The frog AhR still has the similar regions to fish AhR, however, it weakly binds TCDD. The sequence of frog AhR has similar domains like vertebrate AhR except the TAD. More research work is needed to characterise these proteins and perform functional and molecular studies on them (Lustig and Kirsten 1974; Jonsson et al., 2011).

1.10.2 Fish

Fish have high binding affinity of TCDD to their AhRs, mediating extensive environmental toxic effects. The highest effects of dioxin on fish occur during embryonic development (Hahn et al., 1997; Abalos et al., 2010; Kawakami et al., 2010; Zhou et al., 2010). This is why fishes, especially zebra fish, are considered

good candidates to study dioxin effects, mechanisms and functions (Andreasen et al., 2007; Jonsson et al., 2007). On the other hand, it is demonstrated that fish are biologically different to mammals. Fish possess at least two AhR genes, expressing AhR1 and AhR2 proteins. These findings were first discovered in the estuarine killifish, *Fundulus heteroclitus* (Hahn et al., 1997; Powell et al., 2000; Patel et al., 2006), and it is now known that Zebra fish has AhR1 and AhR2 subtypes unlike a single AhR gene in mammals. AhR1 is more similar to mammalian AhR than AhR2, but both AhRs have bHLH, PAS-A and PAS-B domains (Jonsson et al., 2007).

Phylogenetic analysis (Powell and Hahn 2000; Yamauchi et al., 2005; Yasui et al., 2007; Zhou et al., 2010) and gene mapping (Karchner et al., 2002; Evans et al., 2005) showed that fish AhRs are homologues of mammalian AhR. Furthermore, AhR2 seems to be the main AhR protein in fish for binding exogenous ligands (like mammalian AhRs), according to cloning and expression experiments (Karchner et al., 1999; Goldstone et al., 2009; Merson et al., 2009). In general, fish AhR1 and AhR2 have biochemical criteria that are similar to those of mammalian AhR. These molecular properties include dioxin-binding with high affinity, dimerisation with ARNT and AIP interaction with xenobiotic response elements and transcription of a battery of genes mediating the action of dioxins (Law 2001; Finn 2007; Jonsson et al., 2007). However, both transactivation domains of AhR1 and AhR2 are different in structural motifs (Tanguay et al., 2000; Necela and Pollenz 2001; Andreasen et al., 2002) suggesting that the two fish AhRs may have

different functions (Karchner et al., 1999). From the evolutionary point of view, it is supposed that complex gene functions in mammals are split in separate genes in fishes (Evans et al., 2005; Yasui et al., 2007; Zhou et al., 2010). Table 1 summarizes the differences between different AhRs in various species.

Table 1. Comparison of different properties of AhRs in different species.

AhR	C. elegans	Mollusc	Arthropod	Vertebrate1	Vertebrate2	Vetrebrate AhRR
bHLH	yes	yes	yes	yes	yes	yes
PAS domain	PAS-A,B	PAS-A,B	PAS-A,B	PAS-A,B	PAS-A,B	PAS-A,B
Q rich domain	No	yes	yes	yes	No	No
Specific Binding to [H^3]TCDD	No	Nd	nd	yes	yes	nd
Binding to hsp90	yes	Nd	nd	yes	nd	No
Binding mouse ARA9	No	Nd	nd	yes	yes	nd
Dimerizes with ARNT	yes	yes	yes	yes	yes	yes
Binds AhRE	yes	yes	yes	yes	yes	yes
Transcriptional activity	Activator	Nd	Activator	Activator	Activator	Repressor

1.11 Invertebrates

The *Caenorhabditis elegans* (nematode) genome project comparative study showed that *C. elegans* has an AhR ortholog to mammals (CeAhR). Following that, an AhR candidate was detected in the arthropod, *Drosophila melanogaster* (Duncan et al., 1998; Emmons et al., 1999). Recently, several molluscs have been found to express AhR genes (Butler et al., 2001). Invertebrate animals exhibit substantially major differences to vertebrate and mammalian AhRs in that invertebrate AhR is not known to bind dioxins (Hahn 1998). Therefore, the existence of an AhR candidate in invertebrates is a point of interest that explains the evolution of the function of AhR from one species to another.

1.11.1 Drosophila melanogaster

D. melanogaster expresses AhR protein, DmAhR, which is an 884-amino acid sequence. It has, like other AhR homologs, a bHLH domain, which has similar structure to mammalian AhRs. The bHLH is a common structure identity among different types of AhRs and that in DmAhR shares a 71% structural identity with mammalian bHLH. The PAS-B domain of DmAhR shares a sequence identity of 45% with mammalian AhRs (Duncan et al., 1998). DmAhR has a Q-rich domain in its C-terminal half that corresponds to the mammalian transactivation domain, while this feature does not exist in CeAhR. DmAhR interacts with the tango protein (DmARNT) in a yeast two-hybrid assay, and the DmAhR–DmARNT complex can activate a DNA-dependent reporter gene in insect cells that does not require ligands (Emmons et al., 1999). Furthermore, DmAhR protein is not found resting in the cytoplasm, like mammalian or even *C. elegans* AhRs. It is assumed that DmAhR is active without ligands and spontaneously translocates to the nucleus, which may explain the existence of this originally cytoplasmic protein in the nucleus (Butler et al., 2001).

Experimental studies to characterise the function of DmAhR were carried out. A mutated AhR model was created and the animal phenotype showed transformation of the distal antenna into leg structures; the legs were lacking the distal segment, while the bristles were small in size. These structural changes were corrected by restoration of AhR function. Experimental work claimed that DmAhR controls distal-less(dll) (Duncan et al., 1998), the gene that controls flies appendage

maturation (Panganiban 2000), and *bric-a brac* (*bab*) is then regulated by the AhR; *bab* is the transcription factor that controls the maturation of appendages and ovaries. The sexually dimorphic maturation in *D. melanogaster* is indirectly regulated by AhR through the control of *bab* transcription (Kopp et al., 2000; Bunger et al., 2003). The corresponding *DLX* genes in the mammal that have similar function to dll are not known to interact with mammalian AhR. On the other hand, *DLX* genes are responsible for shaping the craniofacial structure and affect teeth maturation. These places are targets for dioxin toxicity in humans (Qiu et al., 1997; Thomas et al., 1997; Hornung et al., 1999; Kattainen et al., 2001; Bunger et al., 2003).

DmAhR and CeAhR play a role in the maturation of chemoreceptive neurons. This invertebrate role of AhR corresponds to the xenobiotic metabolic function of AhR in mammalians. This may be considered an evolutionary advance in the function of AhR from invertebrates to mammals (Duncan et al., 1998; Emmons et al., 1999).

1.11.2 Molluscs

Recently, AhR corresponding genes have been discovered in molluscs; for example, the soft shell clam, *Mya arenaria* (Butler et al., 2001), the zebra mussel, *Dreissena polymorpha* (Hahn 2002), and the blue mussel, *Mytilus edulis* (Hahn 2002). The mollusc AhR homologue contains bHLH and PAS domains like invertebrate AhRs, and is capable of binding the mammalian DNA response

element. Like CeAhR, binding studies failed to demonstrate AhR ligands (Butler et al., 2001).

1.12 Recombinant expression of AhR and AhR-1 for structural studies

Human and mouse AhR has been subjected to recombinant expression. However, the outcome of expression in bacterial systems was completely insoluble AhR (Fan et al., 2009). Full length human and mouse AhR have been expressed in SF9 insect cells. The insect cell system expressed recombinant mammalian AhR in abundant amounts. The expression of AhR LBD was also successful, yet the amount of protein expressed was less than the full length. Studies carried out on rats showed that recombinant AhR could bind ligands including TCDD, however, the major obstacle facing crystallization was the fact that most of the expressed protein was insoluble (Jiang et al., 2009). Co-expression of p23 co-chaperone did not increase the production of the protein or improve its solubility. The major problem was not related to the quantity of expressed AhR protein but the expression of soluble folded mammalian AhR; most of the AhR expressed was insoluble. This makes it extremely difficult to purify enough soluble protein for crystal structure study. The 3-dimensional structure of mammalian AhR has been a major obstacle for many years (Fan et al., 2009).

1.13 Previous AhR and AhR LBD models

Studies of expression of mammalian AhR resulted in non-soluble AhR. It was difficult to obtain a soluble functional AhR for crystal structure analysis, despite the many studies on different expression systems. In order to overcome this problem, computer-based models have been generated to make it possible to understand how AhR binds its ligands. The first trial started 15 years ago by creating a model of AhR utilizing the structure of TCDD as a template for AhR ligands (Waller and McKinney 1995). The weak point of that ligand-based model was because of the ability of the AhR LBD to bind a very wide range of ligands of different structure making this first computer-based model perhaps too speculative. The new theory of AhR modelling depends on the available PAS domain structures. It is known that PAS domain proteins are conservative in structure, despite the diversity of the primary protein structure. This advantage makes any success in discovering a new PAS domain protein structure a breakthrough in the computer modelling of AhR. Any addition of crystal structure will produce a more realistic model. It is thought that the template proteins with 40% similarity and similar biochemical properties have similar structure (Chang et al., 2010; Kikani et al., 2010; Kumauchi et al., 2010; Partch and Gardner 2010).

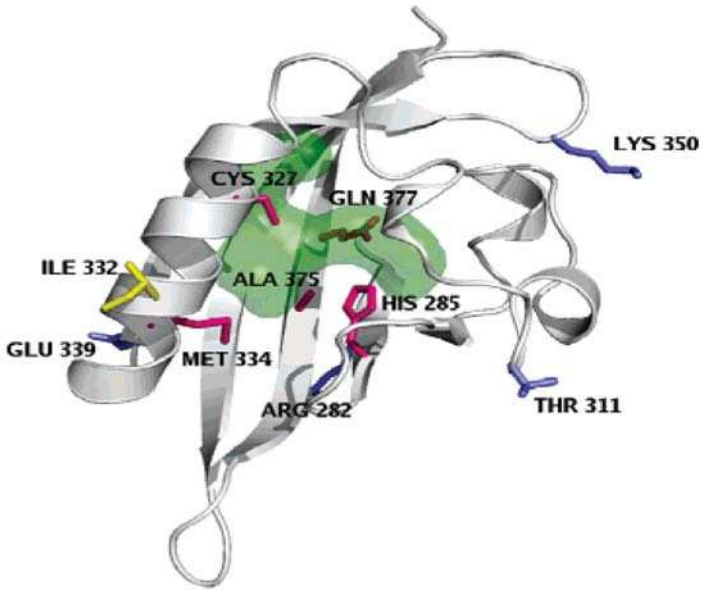


Figure 6. The modeled mouse AhR LBD based on mod_HIF/ARNT as a template. Residues with side chains pointing outside the modelled LBD are blue in colour; residues at the boundaries of the cavity with pink side chains were subjected to mutagensis study; Ile332, is yellow. The green cavity represents the ligand binding domain. The computer-generated model based on these templates was applied using MODELLAR version 8 v1 program. The secondary structure of the AhR LBD was predicted by PSIPRED. The PROCHECK program was used to validate the model. The CASTp server was used to evaluate the AhR LBD pocket (Pandini et al., 2007).

The model shown in Figure 6 is based on template PAS domain structures of HIF alpha2, ARNT, human PAS kinase, human erg potassium channel (HERG), Drosophila clock protein PERIOD, *Adiantum capillus-veneris* chimeric phytochrome/phototropin photoreceptor, mouse receptor coactivator 1A (NCoA), *Bradyhizobium japonicum* sensor protein FixL (FixL) and the *Ectothiorhodospira*

halophila photoactive yellow protein (PYP). These data were obtained from the protein data bank. (Pandini et al., 2007).

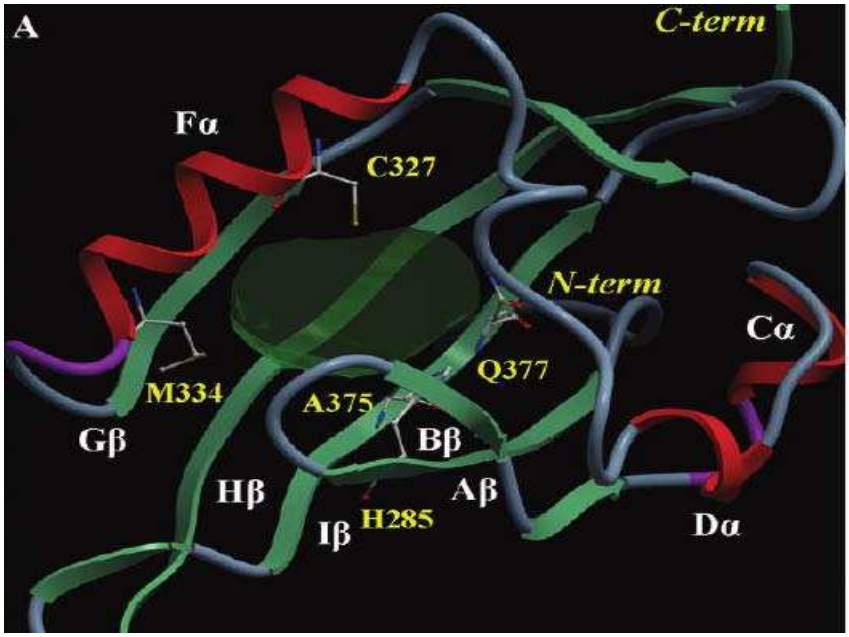


Figure 7. The computer-based mouse AhR LBD modelling performed by Bisson et al. (2009). The green area represents the beta sheets that accomodate the ligands. The ICM function was applied for homology modelling. ICM pocket finder was used to outline the AhR LBD. The modified ECRPP/3 energy function was applied to outline the side chains and the different chemical bonds (Cardozo et al., 1995; Bisson et al., 2009).

The model shown in Figure 7 was constructed based on the available HIF alpha2 PAS domain protein structure existing in the Protein Data Bank (PDB) as a template. The sequence similarity with mouse AhR LBD was about 30%.

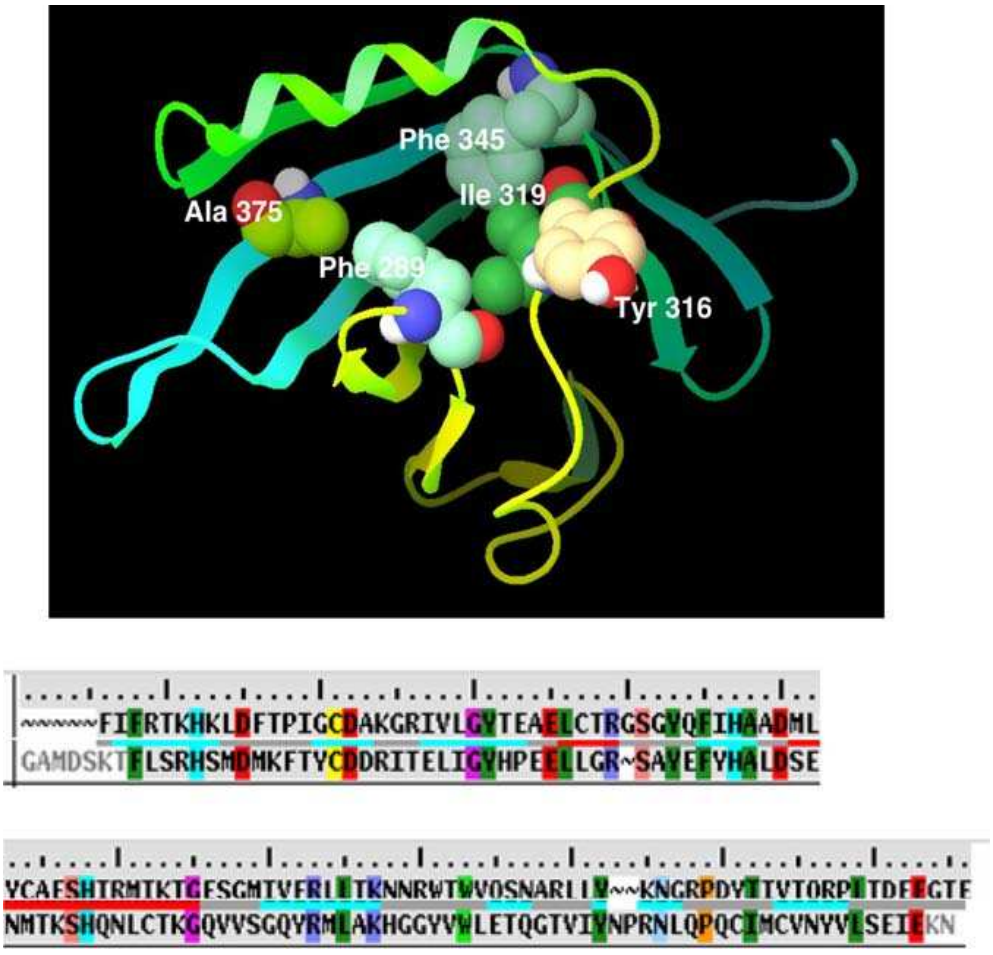


Figure 8. The AhR computer-based model created by Jolalekar et al. (2010) with alignment (below) of mouse AhR (top) and HIF alpha2 (bottom). The highted amino acids are identical between AhR and HIF alpha2. To create this model, Astrex ASP scoring function and the post docking MM-GBSA were applied.

The model shown in Figure 8 was based on HIF alpha2. TCDD and 17 other ligands were investigated by computer-based docking (MM-GBSA). This approach uses a combination of computer-based receptor and ligand templates for

the creation of an AhR LBD model. It is hoped that such models would be more compatible with the real AhR LBD (Jogalekar et al., 2010).

1.14 *C. elegans* AhR

AhR plays a role in the development of invertebrates. Studies showed an essential function in neural development (Vuori et al., 2008). Other studies showed that *C. elegans* AhR-knockout animals have clear neuronal defects in the form of aberrant cell migration and axonal branching. These changes affect neuron differentiation, especially the touch receptor neurone, AVM (Qin and Powell-Coffman 2004).

The *C. elegans* AhR-1 (CeAhR) consists of a 602-amino acid protein that has an overall 38% amino acid identity with human AhR (HsAhR) with the best similarity over the first 395 amino acids (Figure 9) (Powell-Coffman et al., 1998). It is also known to mediate DNA binding, dimerisation with ARNT, and interaction with Hsp90, but is not thought to bind to known mammalian AhR ligands (Powell-Coffman et al., 1998). It is supposed to work as a transcription factor that regulates the development of the nervous system of *C. elegans*. Studies to mutate CeAhR showed that the animals were suppressed in aggregation behaviour. The function was restored when the gene function was expressed back (Qin et al., 2006). The CeAhR protein shares the structural and biochemical properties with mammalian AhR (Figure 9). CeAhR possesses a bHLH domain contained within the specific amino acid sequences that are conserved in mammalian AhR. PAS domains exist

in CeAhR, i.e. PAS-A and PAS-B domains. CeAhR could thus be considered a model for the mammalian AhR.

Figure 9. Comparison between mouse AhR as a representation of a mammalian AhR and the *C. elegans* AhR. The green box represents the AhR ligand binding domain, which shows about 46% similarity with the corresponding domain in the mouse.

Recombinant expression of CeAhR revealed that it is capable of binding ARNT (Powell-Coffman et al., 1998). Interestingly, it is assumed that CeAhR can bind the DNA response element without interaction with xenobiotic ligands. However, these findings could also be seen in mammalian AhR (Dolwick et al., 1993; Jensen and Hahn 2001), fish AhR (Karchner et al., 1999) and other invertebrate AhRs (Butler et al., 2001). On the other hand, CeAhR does not bind AIP in contrast to human AhR (Bell and Poland 2000). The C-terminal half of the CeAhR works as a transcriptional activator; however, its structure is quite different from the corresponding mammalian domain, and it is thought that the PAS domain inhibits the transactivation domain in a consistent manner. Post-translational modification is required for that domain to properly function (Powell-Coffman et al., 1998).

Experimental studies carried out to evaluate the ability of CeAhR to bind ligands showed that recombinant expressed *C. elegans* AhR could not bind either TCDD nor β-naphthoflavone using ligand binding assay (Powell-Coffman et al., 1998). The CeAhR-ligand interaction has been examined by multiple methods. Specific labelling of CeAhR by the photoaffinity ligand, [^{125}I]N_3Br_2DD, could not be detected despite its ability to bind mammalian (Poland et al., 1986; Powell-Coffman et al., 1998) and fish AhRs (Hahn et al., 1994). Other experimental studies using the reversible radioligands [^{3}H] TCDD in velocity sedimentation have evaluated CeAhR ability to bind ligands, but the results were negative. The CeAhR is not known to bind any other mammalian AhR ligands (Butler et al., 2001). The PAS repeat of CeAhR that contains the LBD is very similar to the corresponding human AhR. Most current models of AhR used PAS templates that are less similar to AhR. The success in obtaining the crystal structure of the PAS domain of CeAhR will provide a breakthrough in obtaining a better template to generate a more realistic AhR model, which is more accurate than the published ones. Furthermore, the biochemical properties of CeAhR are closer to those of human AhR than other PAS domain proteins. CeAhR binds human ARNT, and CeAhR can activate the human xenobiotic DNA response element (Huang et al., 2004; Qin and Powell-Coffman 2004). It is logical to say that the best AhR model will be based on a CeAhR template.

1.15 Aim of the study

It is important to say that TCDD toxicity is not only dependent on AhR binding, but may also be related to the ligand position within the ligand binding domain. It is thought that changes in ligand position determine whether agonist or antagonist effects are caused by a specific ligand. So 3-dimensional structure analysis will be very important in understanding the mechanism of action of the AhR receptor and its response to different ligands (Henry and Gasiewicz 2008).

It is vital to understand the binding of ligand to AhR, as it is not well understood even now (Dzeletovic et al., 1997; Vuori et al., 2008). Crystallography and structural analysis of AhR is an important step to visualise how AhR protein works. Unfortunately, trials to express mammalian AhR have failed to express AhR protein in a soluble form that is suitable for crystallography. CeAhR has structural and biochemical properties similar to mammalian AhR (Powell-Coffman et al., 1998). As a result, it is considered a good model for mammalian AhR, and hopefully crystallography will be easier to achieve, allowing 3-dimensional AhR analyses. Therefore, the aim of this study is to express the LBD of CeAhR (the most important part in ligand binding) initially in bacteria or in other systems if this is unsuccessful. The next step will be to purify soluble CeAhR LBD after large scale expression. Finally, attempts will be made to crystallize the protein and submit it for X-ray diffraction studies to determine its structure.

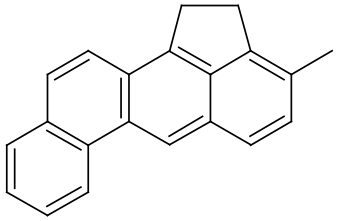
2 MATERIALS AND METHODS

2.1.1 Chemical reagents

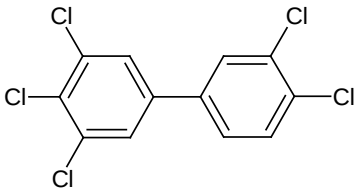
All common chemical reagents were of analytical grade and were obtained from Sigma-Aldrich (Germany), Melford Laboratories (UK) or Fisher Scientific (UK). Yeast extract was from Difco Laboratories (USA), Lysozymes and glycerol were obtained from Courtin & Warner (UK), buffers for DNA digestion were purchased from New England Bio labs (USA), His Binding resin was from Novagen (Germany) and PCR Master Mix and PCR Ready Mix were from GE Health Care (UK).

2.1.2 AhR agonists

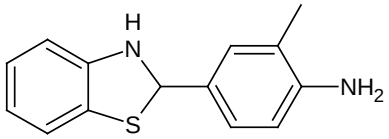
AstraZeneca compound **1c** (AZ1c; 3-hydroxy-2-[4-(trifluoromethyl)phenyl]-[1,2,3]-triazolo[1,5-a]quinolinium hydroxide; Fig. 10) was from AstraZeneca (UK). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), 3MC (3-methylcholanthrene) and PCB126 (3,3`,4,4`,5-pentachlorobiphenyl) (Fig. 10) were from Cambridge Isotope Laboratories. 2-(4-amino-3-methylphenyl)benzothiazole (AMB) (Aylward et al., 2005) and β-naphthoflavone (Fig. 10) were from Sigma.



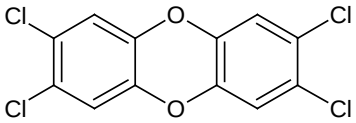
3-methylcholanthrene
(3MC)



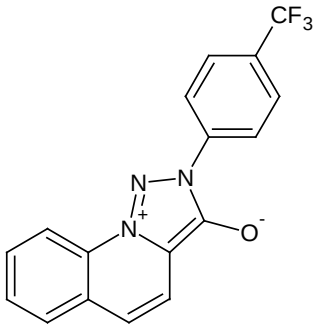
3,3',4,4',5-pentachlorobiphenyl
(PCB126)



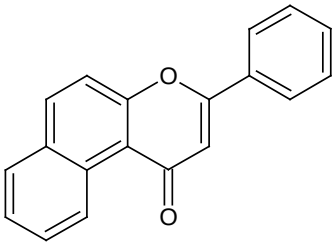
2-(4-amino-3-methylphenyl)benzothiazole
(AMB)



2,3,7,8-tetrachlorodibenzo-*p*-dioxin
(TCDD)



3-hydroxy-2-[4-(trifluoromethyl)phenyl)]-[1,2,3]
-triazolo[1,5-a]quinolinium hydroxide
(AZ1c)



β-naphthoflavone

Figure 10. 2-D structures of the AhR ligands used in lethality tests with *C. elegans*.

2.1.3 Antibodies

Monoclonal anti His tag HRP mouse antibodies were from Sigma (Germany) and anti-GST HRP conjugate was from GE Healthcare (UK).

2.1.4 Enzymes

The enzymes used in this work; zymolase, lysozymes, DNAse1, restriction digestion enzymes (BamH1, Sac1, Nhe1, Xba1, EcoR1, Pvu2, etc.), DNA Ligase, Taq polymerase and RNA reverse transcriptase were obtained from New England Biolabs (USA).

2.1.5 Kits for molecular biology

Miniprep kit, Maxiprep kit, Gel purification kit and DNAse kit were from Qiagen. RNA /cDNA Kit was from Stratagene.

2.1.6 Microorganism Strains

- *E. coli* (PL21DE3, Arctic Express, JM109, SCS111, PO4 50)
- *Pichia pastoris* Strain JM115 Mut+
- *C. elegans* N2
- *C. elegans* GFP 34 A9, 35 A2, 29 A2 strains
- AhR-1 null *C. elegans* (CZ 24 85)

2.1.7 Plasmids and Constructs

- pRSET A containing *C. elegans* AhR LBD
- pPICZ B containing GST *C. elegans* AhR LBD
- pPICZ B containing His Tagged *C. elegans* AhR LBD
- pPICZ alpha B containing His Taged *C. elegans* AhR LBD

- pPICZ alpha B containing GST *C. elegans* AhR LBD
- pPD30.38 containing His Tagged *C. elegans* AhR LBD
- pPD30.38 containing GST Tagged *C. elegans* AhR LBD
- pET-41b containing *C. elegans* AhR LBD
- pGEMT, pGEMT easy Kits
- pPICZ B from Invitrogen
- pPICZ alpha B from Invitrogen
- pET-41b from Novagen

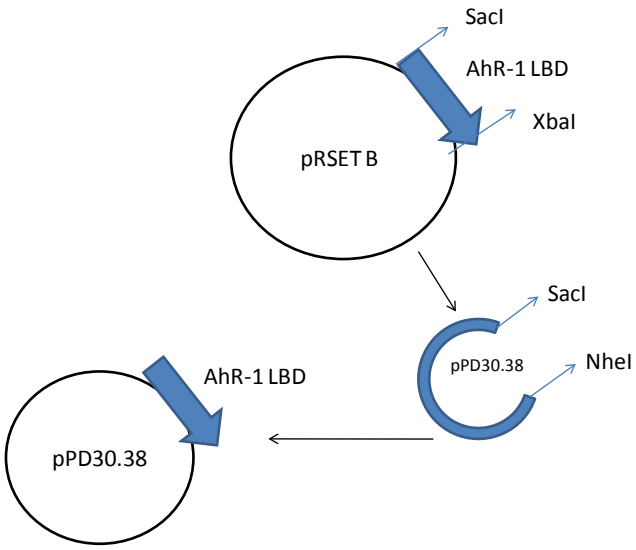


Figure 11. Illustration of the sub-cloning of CeAhR (AhR-1) LBD into pPD30.38 plasmid to create a construct ready for microinjection into *C. elegans* for creating His tagged AhR.

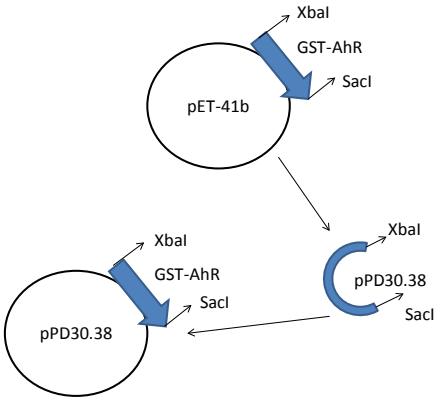


Figure 12. Illustration representing the sub-cloning strategy for creating the GST-AhR construct in pPD30.38 to be microinjected in *C. elegans*.

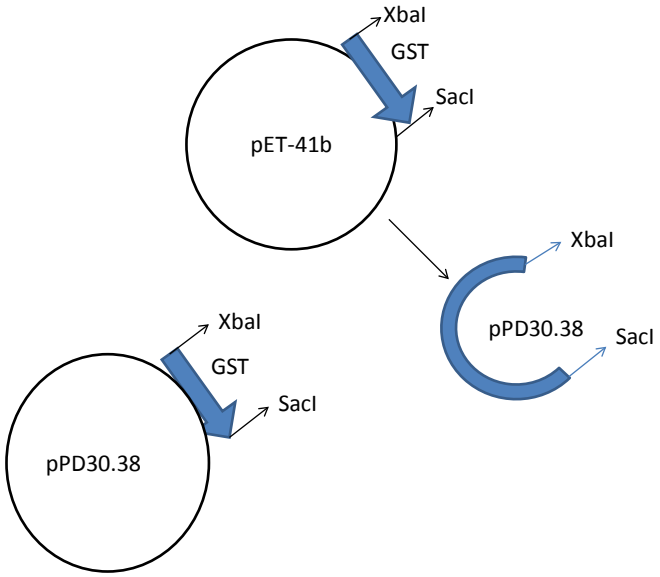


Figure 13. Sketch representing sub-cloning of GST tag into pPD30.38. The figure explains how GST was lifted from pET-41b by both XbaI and SacI restriction digestion enzymes to be inserted into pPD30.38 creating a control construct.

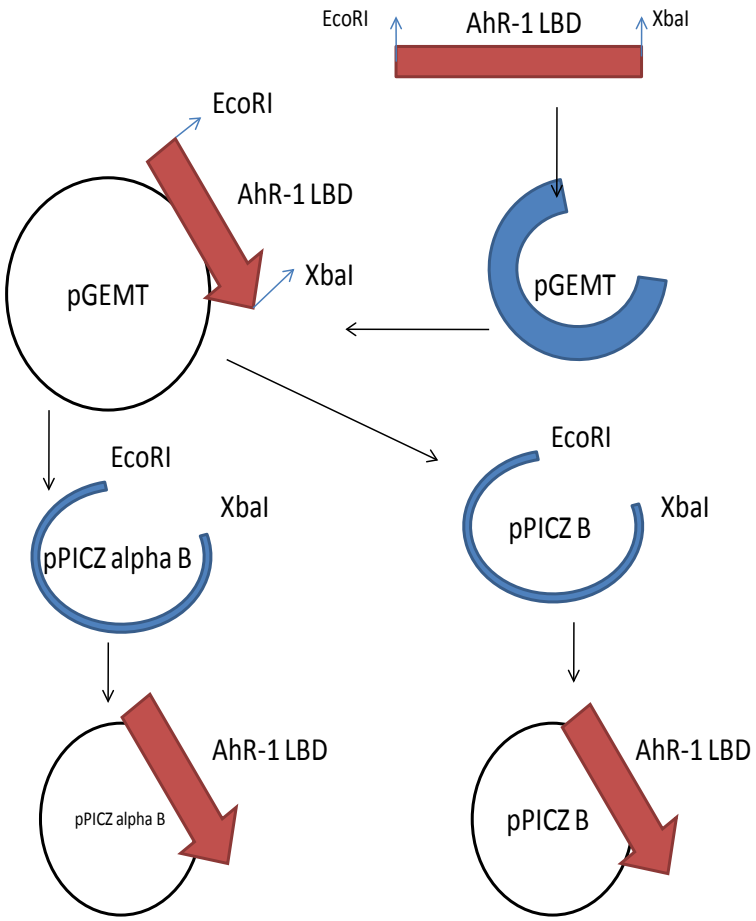


Figure 14. Illustration showing the multiple cloning of CeAhR (AhR-1) LBD with EcoR1 and Xba1 sites in pPICZ B and pPICZ alpha B.

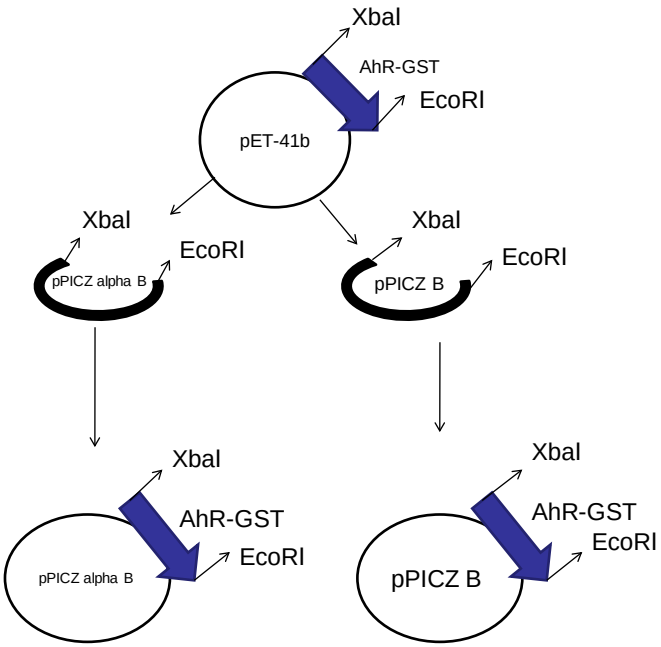


Figure 15. Sub-cloning of AhR-GST. AhR was lifted from pET-41b plasmid to be cloned into both pPICZB and pPICZ alpha B.

2.1.8 Construct design by Vector NTI Suite 7

The DNA constructs were designed by the Vector NTI 7 program for expression of CeAhR LBD protein in the *Pichia pastoris* expression system.

2.1.9 Primers

Table 2. Primers used in this study.

1 - Forward (385) primer for pPD30.38	TGC CAA CAT CCT GCG GA
2 - Reverse (385) primer for pPD30.38	ATA ACA AAA ATA GCG GGT GGG AG
3 - Forward AhR PCR	GA ATT CAC ATG GGA TTT TTG AGA ATT GAC ATG CGC
4 - Reverse AhR PCR	TCT AGA AA TAA TGG AAG TGC AGC TGT TGA TTG GAG
5 - AOX1 forward	GAC TGG TTC CAA TTG ACA AGC
6 - AOX1 reverse	GCA AAT GGC ATT CTG ACA TCC
7 - AhR cDNA forward	CAT GGA TTA CCA TCA TCG TA
8 - AhR cDNA reverse	TGG TAG ATC AGT TTC ATC AA

2.2 Methods

2.2.1 *E. coli* DNA Techniques

2.2.1.1 Preparation of Agar plates with Ampicillin

LB agar (tryptone 10g, NaCl 10g, yeast extract 5g, agar 12g) was added to one litre distilled water (D.W.) and was autoclaved for 20 minutes. The agar was melted in the microwave (15 minutes, 50% power), and then left in a 60°C water bath for 20 minutes. At the same time, ampicillin was put in the same water bath making the temperature of both the agar and the antibiotic the same. After 20

minutes, the ampicillin was added to the agar liquid to a concentration of between 50 to 100 μg/ml.

2.2.1.2 Preparation of chemically competent cells

A fresh plate was streaked to purity with *E. coli* (cell type: JM109) then a single colony was picked up and grown in 5 ml LB broth (10g tryptone, 10g NaCl, 5g yeast extract in 1L distilled water) containing no antibiotic. The cells were left to grow overnight in the incubator at 37°C, with shaking at 220 rpm on the orbital shaker. The following day, the 5 ml culture was added to larger volumes of LB broth, according to the stock needed. An example was adding 5 ml of the cells grown overnight to new fresh 50 ml LB broth that was left in the incubator at 37°C with shaking at a rate of 220 rpm for 3 hours. The OD was measured at 600 nm and the optimum result was 0.6. The cultures were centrifuged at 4000×g for 15 minutes at 4°C. The supernatant was removed and the pellet re-suspended and washed with 50 ml ice-cold sterile 0.1 M $CaCl_2$. The pellet was repeatedly re-suspended and washed in smaller volumes of ice-cold sterile $CaCl_2$; the final volume was a 2 ml suspension. 10% glycerol was added and the volume was divided into sub-aliquots of 200 μl bacteria and kept at -80°C for transformation.

2.2.1.3 Transformation of *E. coli* with PrsetA plasmid containing CeAhR LBD

1μl of CeAhR LBD DNA dissolved in water was added to the thawed, chemically-competent cell aliquot, which was about 200 μl of suspended cells. The DNA was

mixed with the cells and kept on ice for 2 minutes. The following step was to heat shock the mixture at 42°C for 90 seconds, the samples were then transferred to a hot water bath at 42°C then rapidly returned to ice for another 2 minutes. This rapid change in temperature is intended to allow the DNA to pass through the cell membrane of the $CaCl_2$-treated cells. 1 ml of SOC media (20g peptone, 5g yeast extract, 2ml 5M NaCl, 2.5 ml 1M KCl, 10ml 1M $MgCl_2$, 10 ml 1M $MgSO_4$ and 20ml 1M glucose in 1L distilled water) was rapidly added to the cells and the whole mixture incubated at 37°C for about 45 minutes. 200 µl of cells were plated on an agar plate containing ampicillin, and then incubated overnight at 37°C to allow the bacterial colonies to grow.

2.2.1.4 Qiagen miniprep of the plasmid DNA

DNA was re-purified from the colonies plated in the previous step to make sure that CeAhR LBD-containing plasmid has been successfully transformed into *E. coli*. In order to extract the plasmid DNA a Qiagen Miniprep Kit was employed. The experiment followed the kit manual; first, one colony was picked up and inoculated into 5 ml ampicillin-containing (100 µg/ml) LB broth. It was left overnight in the incubator at 37°C with shaking at 240 rpm. The next step was transferring the culture to Eppendorf tubes, and centrifuging at maximum speed (20,000×g) at room temperature for 10 minutes. The pellet was then suspended in 250 µl of P1 buffer and mixed well, then 250 µl P2 buffer was added, and gently mixed by inversion of the tube 4-6 times. 350 µl N3 buffer was then added and the

mixture centrifuged at maximum speed (20,000×g) for one minute. The supernatant was taken and pipetted into the QIAprep spin column. This column was centrifuged again at maximum speed (20,000×g). The column was then washed by adding 750 µl Buffer PE to the column and centrifuging at maximum speed (20,000×g) for one minute. After that, the column was removed and put in a new Eppendorf tube where 50 µl of elution buffer was added and left for 1 minute, then centrifuged at maximum speed (20,000×g) for 1 minute to get a final volume of 50 µl containing the desired DNA.

2.2.1.5 Restriction digestion

The pRSET plasmid DNA was digested by BamH1 restriction digestion enzyme. This enzyme works at 100% activity in Buffer 4. The volume of the reaction was 20 µl of the cocktail including 10% enzyme by volume. 4 µl of DNA was added to the reaction and the mixture volume completed with buffer, BSA 100 mg/ml and water. The reaction was incubated at 37°C in a water bath for one hour.

2.2.1.6 DNA Gel Electrophoresis

50 ml of 1% agarose was prepared by adding 0.5 mg agarose powder to 50 ml TAE buffer (40mM tris acetate, 1 mM EDTA); then the mixture was heated in the microwave oven at 50% power for 1-5 minutes. The solution was checked and found to be clear. The following step was to transfer the solution to a 60°C water bath, then load it onto the gel cassette with suitable combs, and leaving it at room

temperature for 20 minutes until the gel settled. The digested DNA was loaded to the gel with a suitable DNA Marker (1kb plus). The next step was to load the DNA to the combs and run electrophoresis at 80 volts for 45 minutes, with the negative electrode near the comb (DNA) site.

2.2.1.7 pRSET Plasmid amplification

200 µl of chemically-competent *E. coli* cells (JM109) were prepared and transformed with 1µl pure Prset Plasmid containing the CeAhR LBD. The cells were incubated on ampicillin-containing agar plates at 37°C for 24 hours. The following day, one or more colonies were picked up and grown in LB broth containing 50 to 100 µg/ml ampicillin for another day, where the culture was incubated at 37°C with shaking at 220 rpm overnight. The growing cells were mini-prepped according to the Qiagen Kit protocol. The presense of the CeAhR LBD fragment was confirmed by digestion and electrophoresis. The amount of DNA was estimated by the Nano Drop 1000 Spectrophotometer (V 37). At 260 nm wavelength each 50 µg/ml DNA gives 1 absorbance unit. From this, linear comparison with the sample absorbance reflects the amount of DNA in the sample.

2.2.1.8 Ethanol precipitation of DNA

For each volume of DNA, ½ volume of 7.5 M ammonium acetate was added, then double the resultant volume of 100% ethanol was added and the mixture frozen at

-20 °C for at least 1 hour. The sample was thawed then centrifuged at the maximum speed (20,000×g) for 30 minutes. The supernatant was discarded, and the pellet washed with 70% ethanol for one minute, then the sample was centrifuged again for another minute. The remaining fluid was removed keeping the pellet at the bottom of the tube. The pellet was left to dry for 5 minutes and then ultra pure water was added to dissolve the DNA to the appropriate volume of about 10µL.

2.2.1.9 DNA ligation reaction setup

The ligation reaction is an overnight reaction, and is set up using the following:

- 1 µl of 10x ligation buffer from pGEMT kit
- 1µl of DNA ligase from pGEMT kit
- 8 µl of vector/insert mixture.

The ratio between the insert and vector should be at least 3:1. Different ratios were made empirically for best results.

2.2.2 QIAquick Gel Extraction Micro centrifuge Protocol

All experimental work was done at room temperature. 4 times volume of 100% ethanol was added to Buffer PE and the bottle marked accordingly for further use, and a 50°C water bath made ready. The centrifuge was ready at (18,000×g). The protocol to extract the DNA continued by weighing the gel fragments inside the Eppendorf tube, and adding 3 times the volume of Buffer QG. Each 100 mg weight was considered a 100µl volume. The tube containing the gel and Buffer QG was

incubated for 10 minutes in a 50°C water bath. The next step was making sure that the gel had dissolved and that the mixture colour was yellow. Then an equal volume of isopropanol was added to the mixture. The mixture was transferred to the QIAquik spin column in the 2 ml collection tube provided. The sample was centrifuged for 1 minute and the flow-through discarded, then the column was returned to the collection tube, as the DNA was supposed to stick to the column. The maximum volume of the column is 800 µl; so if the sample volume was more than that, then centrifugation was repeated more than once. 0.75 ml of Buffer PE was added as a washing step, and the sample was centrifuged for 1 minute and the flow-through discarded. The column was returned to the collecting tube again. The DNA was left in Buffer PE for 2-3 minutes before centrifugation for the ligation reaction process. After that, the column was transferred carrying the washed DNA to a new clean 1.5 ml Eppendorf tube. 50 µl of Buffer EB (10mM Tris-HCl, pH 8.5) or water was added as an elution step to the centre of the column for 1 minute then the sample was spun for another minute. The purified DNA was measured by the Nano drop machine or subjected to gel electrophoresis for further evaluation. The purified DNA was mixed with 5 times DNA loading dye and added to agarose gel and run for 45 minutes to check for the existence of the purified DNA.

2.2.3 Preparation of electro competent cells

A single colony of *E. coli* (JM109) was picked up from fresh plate, and grown overnight in 5 ml LB broth. The following day, the growing bacteria were diluted

in 50 ml LB and incubated at 37°C with vigorous shaking at about 220 rpm for two hours. The OD, which is supposed to be 0.6 at 600 nm wavelength, was checked and if it was higher it was essential to dilute the sample and incubate it with shaking again until the optimum OD was obtained. It is known that the doubling time for *E. Coli* is 20 minutes.

Following that, the cells were spun in the centrifuge, and re-suspended in the same volume of autoclaved ultra purified water at 4°C. The cells were kept in ice for 10 minutes and spun again before washing with water. Washing was repeated 5 times in the same manner. In the final wash, the volume of water was reduced to 3 times the volume of the pellet that was estimated roughly with naked eye. Fresh electro competent cells could be used for transformation, or 10% glycerol added, and the cells frozen in aliquots for later transformations.

2.2.4 Electroporation of DNA into competent JM 109 cells

The electroporator was set up for *E. coli* using an applied voltage of 1.8 kV. 40 µl of cells were loaded into 1cm^3 volume cuvettes and voltage applied to the DNA until a ring was heard. The time constant was measured by the machine and this should be at least 4 seconds; for clean competent cells, the time constant should be around 5 seconds. As the DNA solution contains salts from buffers that reduce the time constant, it was best to dilute 1 µl of the DNA into 10 µl ultra pure water and another 1 µl of the diluted DNA was taken and mixed with cells in ice-cold cuvettes, before applying voltage to them. Immediately after applying the electric

current, the cells were rescued with 800 µl SOC medium. The cells were incubated in a 37°C water bath for 45 minutes. The next step was to plate the cells on ampicillin agar plates and spread them; each plate carrying 200 µl cells. They were left to dry and incubated for 24 hours at 37°C then checked for growing colonies the next day. The colonies were picked up, grown overnight and mini-prepped as in section 2.2.1.4. The extracted DNA was doubled digested with suitable restriction digestion enzymes to check if the insert was integrated in the new plasmid or not. There were multiple controls that were set up to detect transformation success. First, the electroporated cells without DNA represented the negative control. An ampicillin resistant plasmid with known concentration was subjected to voltage as transformation efficiency control. Transformation efficiency was calculated by counting how many colonies appeared per transformation of 1µg DNA. The electroporation efficiency was supposed to be $1x10^8$ per 1 µg DNA transformed. For example, in transforming 1 picogram of known ampicillin resistant plasmid DNA, it would be expected to find 100 colonies in the plate the following day. This was a positive control that evaluates the success of transformation of DNA inside healthy competent cells. To evaluate the success of the ligation reaction, there were other controls. First, re-ligated, single one end digested CeAhR DNA vector could be transformed to evaluate the efficacy of the ligase enzyme and health of the cloned DNA ends. The double digested vector could be treated by ligase enzyme and transformed as a background control. On the other hand, an agarose gel was run to see the band of

re-ligated DNA, of the size equal to both vector and insert, to make sure that the DNA had been successfully cloned into the selected vector.

2.2.5 Plasmid Maxiprep using QIAGEN Kit

A single colony was picked up and grown in 5 ml LB broth with suitable antibiotic as a selection method for the target plasmid. The sample was incubated at 37°C overnight with vigorous shaking (240 rpm). The 5 ml culture was diluted in another 500 ml LB containing the selective antibiotic for another night. The sample was centrifuged at 6000×g for 15 minutes at 4°C on a JA-10 Beckman rotor. The pellet was re-suspended in 10 ml Buffer P1. The cells were completely re-suspended without remaining debris either by vortex or pipetting up and down. Another 10 ml of Buffer P2 was added and mixed well with cells by inverting the collecting tube or flask 4-6 times; the sample was then left at room temperature for 5 minutes. 10 ml of Buffer P3 was added and gently but rapidly mixed by inverting 4-6 times; this time, the sample was incubated in ice for 20 minutes. The mixture was then centrifuged at the maximum speed (20,000×g) for 30 minutes at 4°C. The supernatant containing the target DNA was separated, and centrifuged again at the same speed for another 15 minutes. Then, the supernatant containing the plasmid DNA was taken off. The gravity column was prepared, and 10 ml Buffer QBT added. The supernatant was filtered through the column by gravity. The column was then washed with buffer QC. The column was loaded twice with 30 ml buffer to wash the entire DNA. The plasmid DNA was eluted by adding 15

ml of Buffer QF. The DNA was precipitated by adding 0.7 volumes of isopropanol and mixing gently at room temperature. Following that, the DNA was spun rapidly at 15,000×g for 15 minutes at 4°C, and the supernatant carefully removed. The pellet was washed with 70% ethanol at room temperature, and the sample spun at 15,000×g for 10 minutes. The supernatant was discarded keeping the pellet at the bottom of the tube. The final step was to leave the DNA pellet to dry for 5 minutes, and then it was dissolved in water or suitable buffer (e.g. 10 mM Tris-HCl, pH 8). The DNA was analysed using agarose gel electrophoresis.

2.3 Protein Techniques in E. coli

2.3.1 SDS-PAGE Gel Electrophoresis

100 ml acrylamide gel mix was made up as follows:

Table 3

Chemical	Running Gel	Stacking Gel
Acrylamide (33%)	% of gel x 3.33 ml	1.3 ml
1.5 Tris-HCl pH 8.8	25 ml	-
0.5 Tris-HCl pH 6.8	-	2.5 ml
10% SDS	1 ml	0.1 ml
10% ammonium persulphate (fresh)	0.5 ml	0.05 ml
TEMED	0.05 ml	0.01 ml
D.W.	to 100 ml	to 100 ml

(TEMED is NNN'N'-tetramethylethylenediamine)

The solutions were filtered and stored at 4°C in the fridge. Fresh 10% ammonium persulphate was added to the remaining solution combination just prior to use. Generally, for each gel, 5 ml running gel and 2 ml stacking gel were needed.

To prepare the gels, clean glass plates with a spacer in between were prepared so as to assemble the gel in the created space. The glass plates should be clean, dry and sealed, so as to retain the gel material in the space between the two plates of each cassette. 100 µl of 0.1 % SDS was poured rapidly to cover the surface of the running gel. Following that, the gel was left to set for 20 minutes at room temperature. After that, the gel was confirmed for polymerisation, and the overlying SDS solution removed. 2ml of stacking gel was prepared by adding 50 µl 10% ammonium per sulphate which was then loaded onto the gel cassette. The stacking gel was supposed to reach near the top of the smaller glass plate. Rapidly, the comb was added to the space between the two glass plates and allowed to set.

2.3.1.1 Electrophoresis

The BIO-RAD container was assembled with either one or two gel cassettes, and immersed in 1x running buffer (composed of 28.8g glycine, 6.04g Tris base, 2g SDS, 1.8 litre dd water). The inner tank should be covered with buffer to the top of the small plate. The combs were removed before sample loading. The protein samples were loaded with 1x or 5x protein loading dye then heated in a 95°C water bath for at least 5 minutes to denature the protein bands. The gel was run for

75 minutes at 100 volts. The following step was to stain the gel with Coomassie or SYPRO Ruby stains. In some experiments, the gels were used for western blotting.

2.3.2 Protein expression in E. coli

Chemically competent transformed PL21DE3 and Arctic Express cells were incubated at 37°C for 24 hours on ampicillin-loaded plates as a method to select *E. coli* colonies that contain Prset Plasmid carrying the gene of interest. The colonies of interest were picked up and left to grow in LB broth loaded with 50 μg/l ampicillin. The samples were incubated overnight at 37°C with strong shaking at 220 per rpm. The following day, the growing bacteria were diluted by 10 to 100 times in LB broth containing ampicillin, and left in the incubator with vigorous shaking for 2 hours. The subsequent step was to measure the OD of the growing bacteria containing the plasmid of expression at 600 nm wavelength; the optimum reading was 0.6. Control bacteria having the anti sense Prset plasmid were grown in the same way, and at the same time. After preparation, induction of the cells was started. 1mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) was loaded to the growing cells, with non-IPTG loaded cells as a control. The volume of the induced samples was expected to be 50 ml or more. If PL21DE3 cells were used, the induction time would be 3 hours in the incubator with vigorous shaking at 37°C or 30°C in different experiments in 2 different shakers. On the other hand, Arctic Express cells were induced overnight at 12°C with vigorous shaking.

2.3.3 Protein extraction and analysis

The samples and controls were removed from the incubators at the correct time, 3 hours for PL21DE3 bacteria and 12 hours for Arctic Express Cells and the samples spun at 20,000×g for 10 minutes; the supernatant was discarded. The cells were re-suspended in a smaller volume of Tris-NaCl Buffer (50mM Tris, 150mM sodium chloride). Generally, each 10 ml culture was re-suspended in 3 ml of buffer. However, if the amount of expressed protein is low, the sample could be re-suspended in a smaller volume of buffer. Then, 1mg/ml lysozyme was added to the buffered cells to lyse the cell wall. The mixture was incubated at 27°C for 30 minutes. The next step was to sonicate the samples to break the cells down. Large volume samples were sonicated for 5 minutes at 60% power for 50% of the time (the sonicator has a timer that produces the ultra sonic wave per time). The samples should be kept in ice as overheating would coagulate the targeted protein. To differentiate the soluble from non-soluble protein fraction, centrifugation at 20,000×g for 30 minutes was applied, and the supernatant kept separate, while the pellet protein was kept as a control. To analyze the samples, 5x SDS protein loading dye was added to 20 µl of the prepared protein either soluble or pellet. The samples were heated at more than 95°C for 5 minutes, then loaded onto acrylamide gel. Electrophoresis was run for 75 minutes at 100 volts with a suitable protein marker. The gel was stained with Coomassie stain for 45 minutes (0.25 g Coomassie R-250 mixed with 90% volume of methanol:water (1:1 v/v) and 10% glacial acetic acid, allowed to mix for one hour and filtered through Whatman

3MM paper), and destained later after 45 minutes with destaining solution (30% methanol and 10% glacial acetic acid in distilled water). Usually the gel was de-stained multiple times every 20 minutes until the bands became clear. The gel was transferred to the gel dryer where it was dried for 1.5 hours.

2.3.4 SYPRO Ruby dye for protein gel staining

SYPRO Ruby is a very sensitive stain that is supposed to detect as little as 1 ng protein bands separated by electrophoresis. It was used as an alternative to Coomassie to detect small quantities of protein. Once electrophoresis was finished, the gel was immersed in 50 ml of SYPRO Ruby stain (the stain should kept away from light in a clean box covered with aluminium foil). The gel was kept in stain for at least 3 hours or overnight. In the following step, the gel was de-stained with solution containing 10% methanol and 7% glacial acetic acid for one hour. The gel was placed into the gel doc machine for direct transilluminating and photographing using the Versa Doc Imaging System Model 1000 from BIO-RAD.

2.3.5 SDS-PAGE Gel Drying

The gel taken from the BIO-RAD cassette after electrophoresis was covered by SARAN film and placed over wet Whatman 3MM paper. The gel was placed in a Model 583 Gel Dryer. The gel was put under vacuum at a temperature of 80°C for 1.5 hours.

2.3.6 Protein purification by histidine-binding Nickel Column

A 20 ml syringe was loaded with 10 ml His-binding resin from Novagen. 30 ml D.W. was used to wash ethanol from the resin. 50 ml 1x charge buffer (50 mM $NiSO_4$) was loaded to charge the resin. D.W. was used to wash out excess $NiSO_4$. 30 ml 1x binding buffer (20mM sodium phosphate buffer, 500mM NaCl, 5mM imidazole) was loaded. The protein sample was loaded at a slow rate (0.25 ml/min). After that, 50 ml of 1x binding buffer was added to remove unbound protein. In the next step, 50 ml 1x washing buffer (20mM sodium phosphate buffer, 500mM NaCl, 30mM imidazole) was added to get rid of weakly attached protein. Finally, the protein was eluted by 30 ml 1x eluting buffer (20mM sodium phosphate buffer, 500mM NaCl, 500mM imidazole).

2.3.7 Protein sequencing of CeAhR LBD band

The expected soluble CeAhR LBD band was cut from the gel and put in an Eppendorf tube and sent for sequencing. The acetic acid percentage in the Coomassie stain and destain should not exceed 4%. The de-staining time should not be more than 45 minutes.

2.3.8 Detection of the CeAhR LBD protein amount

The soluble AhR protein was run on a gel against serial bovine serum albumin (BSA) dilutions. The amount of CeAhR LBD protein was determined by comparison of the thickness of the target band in relation to gradient BSA bands.

Calculations were performed to obtain the CeAhR LBD protein production per litre culture.

2.3.9 Bradford assay

This is a colorimetric method to quantify total protein samples. 5X Bradford dye was formed by dissolving 100 mg Serva blue-G250 in 50 ml 95% ethanol and 100 ml 85% phosphoric acid. De ionized water was added to make the volume of the solution to 1 litre. Serial dilutions of BSA from 0 to 100 μg/ml were freshly prepared from stock solution. To prepare 1X Bradford dye, the 5X dye was diluted in distilled water and filtered through Whatman filter paper. 1 ml fresh dye was added to each of the serial BSA protein concentrations in 20μl volume. Then the sample was vortexed and left for less than 5 minutes at room temperature creating a spectro-photometric standard curve. The sample absorbance was measured at 595 nm. The unknown samples were compared to the standard curve to calculate the concentration of the target samples.

2.3.10 Dot Blot Protocol

This is an antigen-antibody technique to detect proteins. It works on the same principles as Western blotting but without electrophoresis. The protein was identified in situ as a circular spot. The Dot Blot could be used in a semi-quantitative way to evaluate the amount of expressed protein. To perform a Dot Blot, a small strip (1x4 cm) of nitrocellulose membrane was cut. A grid (5mm) was

marked out with a pencil, where blotting was applied. 2 µl of the protein sample was dropped in the centre of the grid. The membrane was left to dry for 5 minutes. Then, the membrane was blocked for non-specific binding sites by 5% BSA in TBS then incubated with anti His tag (0.5 µg/ml) or anti GST HRP conjugate (0.5 µg/ml) for 45 minutes. The following step was to wash the membrane with TBS-T (Tween TBS) 5 times for 5 minutes each time. Finally, the membrane was incubated with ECL reagent (the kit containing the chemiluminescent substance transformed into a light and heat producing product) for 1 minute in the gel doc machine, and the florescence of the protein was detected by the gel doc camera.

2.3.11 Western blot

The protein sample was run by SDS-PAGE gel as mentioned before. Following that, the protein was transferred or blotted to the nitro cellulose membrane. The nitro cellulose membrane was placed on top of the gel then this was sandwiched between 2 pieces of 3MM Whatman paper. The layers were kept as follows: 1- the black edge of the cassette down. 2- One layer of 3MM Whatman paper. 3- The gel. 4- The nitrocellulose membrane. 5- One layer of 3MM Whatman paper. 6- The edge of the cassette. The cassette was put under gentle pressure to remove the air bubbles. This sandwich could be set up under water to remove any air bubbles. The cassette was placed into a Bio Rad tank that was filled with 1X transfer buffer (36g tris, 150g glycine , 4g SDS in 1L distilled water) . The electric current was applied for 1 hour at 90 volts.

After finishing the blotting, the nitrocellulose membrane was moved to a container and blocked with 5% BSA-TBS blocking solution overnight. The following day, the nitrocellulose membrane was incubated with either anti- His HRP tag or anti-GST HRP for 45 minutes with a dilution of 1:10,000 in 5% TBS-T as was done in dot blotting. Then, the nitrocellulose was washed with TBS-T for 5 minutes 5 times. The following step was adding ECL to the nitro cellulose membrane in the gel doc machine. Finally, the machine was closed to detect the fluorescence reflecting the bands of the target protein.

2.4 Yeast techniques

2.4.1 Sub-cloning of GST tagged CeAhR LBD into pPD30.38

The GST CeAhR LBD was lifted from PET41b by double digestion with both Xba1 and Sac1. The CeAhR LBD was inserted into the pPD30.38 vector that was double digested by Nhe1 and Sac1. In order to prevent methylation of DNA that interferes with restriction digestion by Xba1, the plasmid of interest was transformed in SCS E. coli 110. Then the samples were mini-prepped. The DNA was purified and cut with Sac1 and Xba1. The sub-cloning was performed by lifting the CeAhR LBD DNA fragment from the PET41b vector and inserting it into pPD30.38i. The GST CeAhR LBD is 1529bp; 480 amino acids and the expected protein size is about 54 kDa. The expected expressed His tagged CeAhR LBD lifted from Prset is supposed to be 29 kDa, and about 600 bp. The sub-cloned constructs were sequenced using both 385 primers (Table 2).

2.4.2 Cloning into pPICZ alpha B and pPICZ B

GST CeAhR LBD was lifted from PET41b and sub-cloned into both pPICZ B and pPICZ alpha B in a similar way as that sub-cloned in pPD30.38, except that GST CeAhR LBD in PET41b was double digested with Ecor1 and Xba1 and sub-cloned into both pPICZ alpha B and pPICZ B after double digesting the vectors with the same restriction digestion enzymes.

2.4.3 PCR of CeAhR LBD with new restriction digestion sites carrying both Ecor1 and Xba1.

PCR primers 3 and 4 (Table 2) were used to clone CeAhR LBD from the pRSET Plasmid to be sub-cloned into the PGEMT vector.

The PCR reaction mixture consisted of:

1- 30 μl PCR master mix.

2- 1ul each of primers (3, 4)

3- 1 μl DNA (pRSET/CeAhR LBD) at different dilutions

The setup for the PCR program was:

1- 94°C for 30 seconds

2- Annealing temperature: 60°C for 20 seconds

3- Extension temperature: 72°C for 1 minute.

4- Melting temperature: 94°C for 15 seconds.

2.4.4 Sub cloning of CeAhR LBD into pGEMT plasmid

The CeAhR LBD ligation reaction was setup in pGEMT vector. The reaction was formed of 10 µl volume, which contained 1µl ligase, 2µl insert, 2 µl vector and 5µl 2x Buffer. As before, various insert:vector ratios were used. The ligated DNA samples were then transformed into JM109 cells by electroporation. Different controls were set up according to the Promega manual. The reaction was also performed with just cells as a negative control, as well as a transformation efficiency control, a background control and a new positive control represented by the control insert, i.e. blue/white colony selection. The white colonies were supposed to have the ligation constructs. The blue white selection depends on the basis that non construct containing colonies are able to metabolize the substrate on the plate giving the blue colour, however, successfully cloned bacteria contain plasmids that are interrupted by the constructs and therefore not able to metabolize the substrate to produce colour giving white colonies. The ligation reaction was deemed to be successful if the white colonies represented more than 60% of all colonies. As such 6 white colonies were picked up and mini-prepped to detect the CeAhR LBD insert.

2.4.5 Sub cloning of CeAhR LBD from pGEMT into pPICZ alpha B and pPICZ B

The CeAhR LBD PCR product, which has both EcoR1 and Xba1 sites, was cloned in PGEMT. Then, the CeAhR LBD pGEMT construct was double digested

with both EcoR1 and Xba1. The AhR was re-sub-cloned in both pPICZ alpha B and pPICZ B using the same method of cloning using electroporation. The suspected transformed colonies were mini-prepped and double digested again with EcoR1 and Xba1 again to detect CeAhR LBD inserts. So, after multiple sub-cloning CeAhR LBD and GST CeAhR LBD were sub-cloned into both pPICZ B and pPICZ alpha B. In order to check the integrity of the 4 prepared constructs they were sequenced using AOX1 forward primer.

2.5 Electroporation of Pichia

Electroporation is a very good method for isolating multi-copy clones. The first step was growing 5 ml *Pichia pastoris* GS 115 in a 50 ml conical falcon tube at 28 to 30°C. The following day, this culture was added to 500 ml YPD media (10g yeast extract, 20g peptone and 20g dextrose dissolved in 1 L distilled water and autoclaved for 20 minutes) in a 2 litre flask, and incubated overnight until the OD at 600 nm was up to 2. The cells were spun at 1500×g at 4°C for 5 minutes then re-suspended in 0.5 litre ice-cold water. The samples were spun again in the same way, and again re-suspended in 0.25 litre ice-cold water. The samples were centrifuged for a third time and re-suspended in 20 ml of ice cold 1M sorbitol. The samples were spun again for the last time, and re-suspended in 1.5 ml ice-cold 1M sorbitol. The cells were kept in ice for electroporation. The cells should be subjected to electroporation on the same day, as freezing cannot keep the cells competent.

80 µl of the cells were prepared using the above method, and incubated with the ethanol-precipitated linearised target DNA (pPICZ alpha B, pPICZ B carrying CeAhR LBD). The DNA was linearised by digestion with Sac1 restriction digestion enzyme. The mixture was kept in a cuvette in ice for 5 minutes. The Pichia was pulsed at a voltage of 2 kV. The cells were immediately rescued with 1ml ice-cold 1M sorbitol, and transferred to a sterile 15 ml tube. The tube was incubated at 30°C in the incubator without shaking. The next step was plating each 100 µl of cells on individual YPDS Agar plates (10g yeast extract, 182.2g sorbitol, 20g peptone and 20g agar in 0.9 L D.W; 100 ml of filter sterilized 20% Dextrose was added to complete the total volume to 1 L) loaded with 100 µg/ml Zeoicin after cooling the fluid to 60°C. The plates were left in the incubator for 2-4 days waiting for the colonies to reform.

2.5.1 Yeast Genomic DNA Extraction

A yeast colony was picked and grown as a 1.5 ml liquid culture overnight at 30°C in YPD media (10g yeast extract, 20g peptone and 20g dextrose dissolved in 1L D.W. and autoclaved). The following day, the cells were spun at 20,000×g for 5 minutes, and then 200 µl lysis Buffer (2 ml Triton X -100, 5ml 20% SDS, 2 ml 5M NaCl, 2 ml 1M tris-HCl pH 8, and 2ml of 0.5M EDTA made up to 100ml with distilled water) was added. The tube was dropped in liquid nitrogen for 2 minutes, then transferred into a 95°C water bath for 1 minute. This step was repeated and the sample vortexed for 30 seconds. Then 200 µl chloroform was added and the

sample was vortexed for 2 minutes. The sample was centrifuged at 20,000×g at room temperature for 3 minutes. The upper aqueous phase was transferred to an Eppendorf tube carrying 400 µl ice-cold 100% ethanol. The sample was mixed by inversion, incubated at room temperature for 5 minutes, then centrifuged at room temperature for 5 minutes at 20,000×g and the supernatant removed with a pipette. The pellet was washed out with 0.5 ml 70% ethanol. The sample was centrifuged again, and the supernatant was removed. The pellet was air dried for 5 minutes, re-suspended in 10 µl water and the concentration determined (Figure 16).

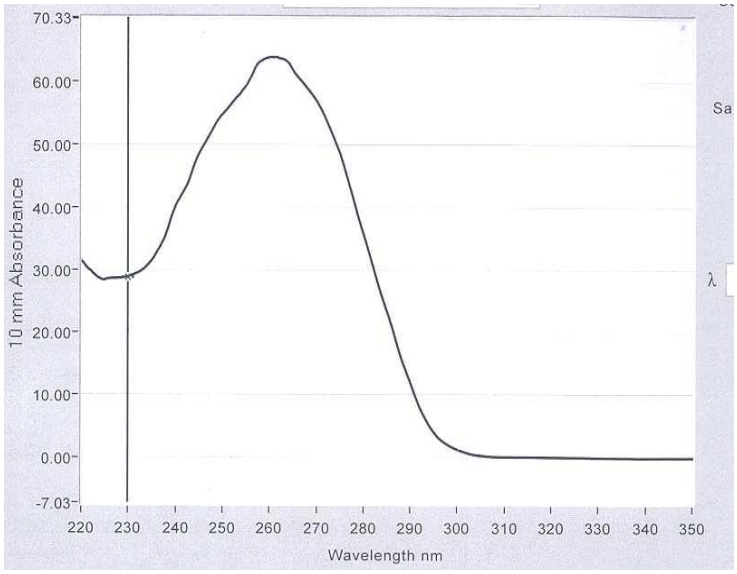


Figure 16. Spectrophotometric curve of DNA (genomic DNA extracted from *Pichia pastoris*) using the Nano Drop machine. The figure shows a graph of the absorbance of DNA sample against wavelength. The software converts the absorbance into DNA concentration (ng/µl). The calculation is based on the equation that 50 ng double stranded DNA gives 1 absorbance unit at 260 nm. The volume of DNA sample is 1µL.

2.5.2 Analysis of Pichia colonies by PCR

To set up the PCR reaction, Pichia genomic DNA was extracted and used as a template for the PCR reaction.

The PCR reaction was set up, with the following required:

1- PCR simple Master Mix, 27 µl.

2- AOX1 forward primer (10pmol/µl)

3- AOX1 reverse primer (10 pmol/µl)

Both primers were mixed and 1µl of the mixture used in each reaction

4- Extracted Pichia genomic DNA, 1 µl containing 0.5 to 1 µg DNA.

The mixture was placed in the PCR machine and the program was set up as follows:

1- Incubation at 94°C for 2 minutes

2- Denaturation at 94°C for 1 minute

3- Annealing at 54°C for 1 minute

4- Extension at 72°C for 1 minute

5- Final extension at 72°C for 7 minutes.

2.5.3 Hot Phenol Yeast total RNA Extraction

This method could be used to extract RNA from 10 ml yeast culture. The cells were collected and centrifuged, and the pellet snap-frozen and stored at –80°C. This method is expected to yield up to 500 µg RNA. The pellet was then collected and re-suspended in 400 µl AE Buffer (50 mM sodium acetate and 10mM EDTA),

then 40 µl 10% SDS was added and the mixture vortexed for 20 seconds. 500 µl phenol was added and the mixture vortexed again for 20 seconds. The mixture was then taken and put in a water bath at 65°C for 4 minutes, after which it was dropped in liquid nitrogen for less than a minute to form crystals. The mixture was thawed then vortexed and the samples were refrozen in liquid nitrogen. The sample was thawed and spun at 4°C for 10 minutes at 20,000×g. The aqueous component was transferred to a fresh tube. An equal volume of phenol was added and the mixture vortexed for 20 seconds and spun at 4°C at the maximum speed (20,000×g) for 10 minutes. The aqueous component, whose volume was about 400 µl, was transferred to another fresh tube. 0.1× volume of sodium acetate pH 5 and 2.5× volume of 100% ethanol were added. The mixture was incubated at -20°C for at least one hour, or even better overnight. Following incubation, the sample was spun at the maximum speed (20,000×g) for 20 minutes and the pellet was washed with 0.5 ml 70% ethanol that was prepared by adding DEPC water to 100% ethanol. The following step was re-suspending the pellet in 200 µl DEPC water and then the RNA concentration was measured on the Nano drop machine and stored at –80°C.

2.5.4 RNA Electrophoresis Gel

1% agarose was prepared as mentioned before in section 2.2.1.5. After melting the gel, 1ml 1% SDS was added to the solution, which was poured in the cassette. The RNA was loaded with 10X RNA loading dye. The samples were run by

electrophoresis for one hour at 70 volts. A DNA ladder was run beside the sample, but in consideration that RNA is single stranded and DNA is double the size of RNA.

2.5.5 RNA to cDNA reverse transcription

This RNA to cDNA kit contains all Buffers and materials required for reverse transcription of total RNA into single stranded DNA in a 20 µl volume reaction. To achieve success in this experiment, the RNA was cleaned and freed from RNase activity. 10 µl 2X RT Buffer were added to 1µl 20X RT Enzyme Mix and made up to 20 µl by adding RNase free water. The sample was mixed well and put in a suitable PCR tube. The tube should not contain any air bubbles, and if so, the sample spun down to remove these air bubbles. The thermal cycler program was set for 3 steps. The first step was 37°C for 60 minutes, the next step was 85°C for 5 minutes and finally the sample was kept at 4°C until it was collected.

2.6 C. elegans Techniques

2.6.1 Sub-cloning of CeAhR LBD into pPD30.38

The first step was double digesting pPD30.38 with SaC1 and Nhe1 restriction enzymes. The success of double digestion was confirmed by running an agarose gel with uncut pPD30.38 as a control, and the single digested pPD30.38 as the other control. At the same time, the CeAhR LBD was lifted from the pRSETb plasmid by double digestion with Xba1 and Sac1 enzymes, and the fragment

inserted in the cut pPD30.38. The insert to vector ratio should be at least 3:1 for a successful ligation reaction. DNA gel electrophoresis of the CeAhR LBD insert and flanked pPD30.38 vector was run at 80 volts for 45 minutes with a suitable DNA ladder (1 Kb +). It was better to post-stain the gel with ethidium bromide keeping the background of the gel clear. The following step involved visualising the DNA bands in the dark reader, where the CeAhR LBD band was cut with a clean razor, and at the same time the flanked pPD30.38 was also cut, and both gel fragments put in clean Eppendorf tubes.

2.6.2 Maintenance of the C. elegans worms

C. elegans were maintained on lawns of OP50 *E. coli* growing on NGM agar plates. The bacteria (200 µl volume) were plated on the NGM agar poured in petri dishes (9 cm) and left in the incubator at 37°C for 24 to 48 hours. Once the *C. elegans* had multiplied and exhausted the bacterial food supply it was necessary to passage them to a new plate. They were transferred from the old plate by "chunking" whereby a piece of agar carrying at least 5 animals was cut out and transferred to the new plate. The cutting of the agar was performed with a sterile scalpel. To make the scalpel sterile, it was heat flamed with alcohol. To prepare NGM plates, 3g NaCl, 17g agar, and 2.5g peptone were added to 800 ml D.W. and autoclaved for 25 minutes. The NGM agar was left in a 60°C water bath. 300 µl of each of 1M $CaCl_2$ and 1M $MgSO_4$, 25 ml K phosphate buffer (250 ml 1M KH_2PO_4/200 ml 1M K_2HPO_4) and 5 g Cholesterol were added to NGM agar

solution in the water bath. The agar complex was poured into 9 cm plates and left to set.

2.6.3 Preparation of *C. elegans* Liquid Culture

On day one, OP50 Bacteria were grown overnight in 1 litre LB broth. 5 ml of already grown bacteria were inoculated into the entire 1 litre of LB. On the following day, the growing bacteria were centrifuged at 2000×g for 20 minutes. At the same time, 1 litre of S media was prepared by adding 2 ml Cholesterol (5g/ml), 2 ml of potassium citrate buffer (20g citric acid monohydrate, 293.5g Tri-potassium citrate, and D.W. up to 1L.), 300 µl 1M $CaCl_2$, 300 µl 1M $MgSO_4$ and 0.5 ml trace elements (1.86g disodium EDTA, 0.69g $FeSO_4.7H_2O$, 0.2g $MnCL_2.2.4\ H_2O$, 0.29g $ZnSO_4.7\ H_2O$, 0.025g $CuSO_4.5\ H_2O$ and H_2O to 1L.) to 1 litre S-Basal (5.84g NaCl, 43.4ml 1M KH_2PO_4, 6.6ml 1M K_2HPO_4, to 1 litre with water and autoclaved). The bacterial pellet was added to about 500 ml S-Media and the OD measured at 600 nm taken. The OD should be 1.7 giving indication that the bacterial food is enough for the liquid culture to grow the animals for up to 6 days. Finally, an NGM plate carrying *C. elegans* worms growing for 2-4 days to give a large number (thousands) that is expectected to grow to millions in the liquid culture was washed with S-Basal, added to the flask containing the S-medium/OP50 mixture and incubated at 20°C with shaking at 200 rpm. The flask was left in that environment for 3-6 days.

2.6.4 Egg bleach to produce synchronized animal stages for lethality test

The worms, grown from the liquid culture, were left to settle in the bottom of a 500 ml bottle, and left for 1 hour in ice. Once the worms collected in the bottom of the bottle, the excess medium was removed carefully from the worm surface. The worms were collected in a 50 ml falcon tube. The same volume of bleach as the volume of the worms was added and continuously mixed for 4 minutes. The sample was centrifuged in a horizontal centrifuge at 500×g for one minute. The eggs and debris were collected in the bottom of the tube. Rapidly and carefully, the extra fluid was removed by a syringe and replaced by S-basal and the sample mixed and re-centrifuged. This was considered one wash. The wash was repeated 4-5 times to remove the remnants of the bleach that could kill the eggs. After the final wash, the sample was mixed in 5 ml of S-Basal and the sample was separated into a 6 well plate, and left overnight so that the eggs hatch into larvae. The animals were spread on new NGM plates and incubated in a 15°C incubator for 3 days before use in the lethality test.

2.6.5 Lethality test for AhR different agonists

The NGM plates carrying uniform L4 N2 *C. elegans* were washed with S-Basal and the animals collected in a 20 ml flask. The supernatant was taken off leaving the worms in a volume of 5 ml at most. OP50 bacteria were collected and spun down, and then re-suspended with S-Basal at OD of 0.7 at 600 nm wavelength. The following was added to each well of a 6 well plate: 900 µl S-Basal, 50 worms,

1µl test compound dissolved in DMSO (Table 5) and 100 µl bacteria. Six different chemicals were examined by lethality tests (see Table 5).

Table 4. The chemical concentrations used in the lethality tests. The chemicals were dissolved in DMSO and 1µl of the solution was taken and diluted in 1ml volume of fluid containing the animals.

Compound	Stock in DMSO	Range used in dose-response experiment
TCDD	1 mM	1nM - 1 µM
AZ1c	1 µM	1 pM - 1 nM
PCB 126	10 mM	10 nM - 10 µM
3MC	20 mM	20 nM - 20 µM
2-(4-amino-3-methylphenyl) benzothiazole	100 µM	100 pM - 100 nM
β-naphthoflavone	300 mM	300 nM - 300 µM

The samples were mixed and the 6 well plate covered by paraffin film and put in a humid box that was closed. Finally, the box was moved to a 15°C incubator for 3 days. The animals were checked every day for 3 days, and after this, the lethality of target toxins was evaluated. The maximum final concentration was diluted 10 fold for 3 times to create a dose response curve. For example, TCDD concentrations used in the lethality test were 1µM, 0.1 µM, 0.01µM and 0.001µM.

2.6.6 Making Protein Gel Samples from Worms

Up to five 9cm plates of worms were grown for 6 days. This was expected to give ~200 µl of packed worms after the washes. The worms were washed off the plates by adding 1ml S- Basal medium on the plate, swirling gently, and the liquid

sucked off with a Pasteur pipette. The worms were spun by centrifugation to pellet the worms, and the supernatant was discarded. The worms were washed once with S-Basal, spun down and the supernatant discarded. The worms were transferred into a small amount of S-Basal in an Eppendorf tube, and centrifuged for a short period at 3,000 rpm, and as much liquid as possible removed. At that point, the worms were frozen at -80°C. Then 1 ml gel sample buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% beta-mercaptoethanol, 12.5mM EDTA and 0.02% bromophenol blue) was added and boiled in the tube (a screw cap Eppendorf was used) for 5 minutes, then the tube was centrifuged for 10 min to pellet the debris and the supernatant transferred to a new tube. Boiling was expected to break the body wall of the adults; sonication for 20 seconds was applied using a Branson sonifier with a probe sonicator having a microtip. The machine was set up at the microtip limit, on a 2 second 50% cycle. Once sonication was achieved correctly, no frothing was detected. By examination of the tube under the dissecting microscope, almost no worms or debris were visible. The tube was centrifuged for 10 min to pellet the debris and the supernatant transferred to a 1 ml screw cap tube. The samples were run by SDS PAGE gel and stained with Coomassie stain.

2.6.7 Measuring the fluorescence of GFP-CYP fused C. elegans

The transgenic C. *elegans* containing GFP-CYP fused enzymes were tested with TCDD, AZ1c, 3MC, PCB 126 and 2-(4-amino-3-methylphenyl) benzothiazole to detect induction of CYP35A2, CYP29A2 or CYP34A9 enzymes that are

conjugated with GFP protein. These CYP enzymes are known to be induced by the xenobiotic response in *C. elegans* (Menzel et al., 2001). The samples were loaded onto a plate reader and a WALLAC 1420 VICTOR^2 was used to measure the amount of the fluorescence in each sample or control. The cocktail samples of animals with added toxins were collected after 3 days of lethality testing. 300µl is the maximum capacity of the each well of a 96 well dark reader plate. The animals were collected from the bottom of the well of the six well plate as used in lethality tests. The samples were transferred to the dark plate with the negative control samples. The plate was placed in the dark reader and the software would read the fluorescence emitted from each plate 4 times. The samples were compared to the controls and the Data were collected in an excel spreadsheet.

3 RESULTS

3.1 Expression of CeAhR LBD protein in E. coli

The CeAhR LBD has been successfully cloned by Dr David Bell in Prset plasmid with BamHI sites. The first approach was to try and express this DNA in *E coli.* Successful expression would yield CeAhR LBD protein of about 29 kDa with a poly histidine tag that would help purification later on.

Experiments to express His-tagged CeAhR LBD protein in *E. coli* BL21(DE3) cells were performed. BL21(DE3) cells were transformed with pRSET plasmid containing His-tagged CeAhR LBD, and with an identical plasmid containing CeAhR LBD in the anti-sense orientiation as a negative control. The cells were induced by IPTG and incubated for 3 hours at 37°C in the shaker and total protein samples isolated. The SDS-PAGE results (Figure 1) showed that cells transformed with both sense and anti-sense express similar protein bands, except for thick protein bands in the sense samples of about 29 kDa. This band corresponds to the expected size (CeAhR LBD) is 200 amino acids which is 29 kDa, of the predicted protein for the His-tagged CeAhR LBD.

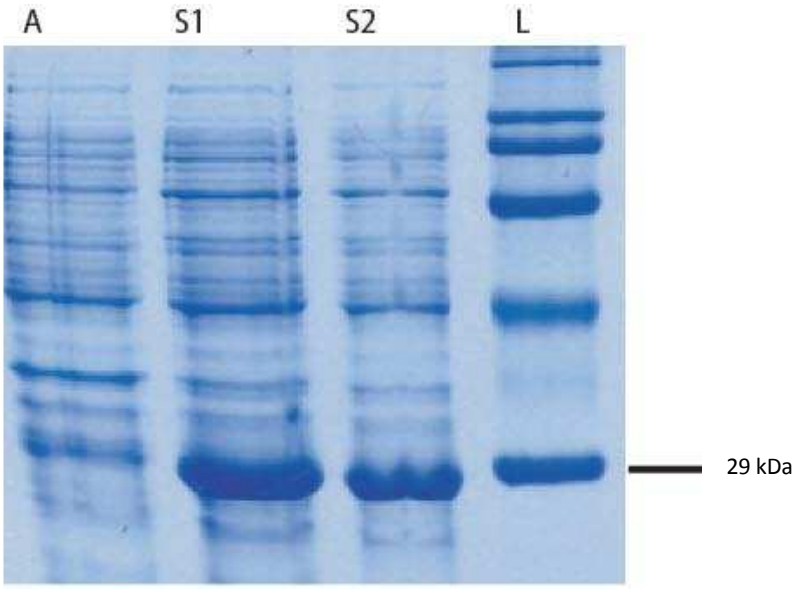


Figure 17. SDS-PAGE gel of total bacterial extracts. BL21(DE3) bacteria were transformed with pRSET plasmid containing the AhR LBD in anti-sense (A) or sense (S) orientation. Cells were induced for three hours with IPTG at 37°C, and total protein isolated. The samples consist of 10 µl of PL21(DE3) cells transformed with anti-sense histidine-tagged CeAhR LBD (A) or sense histidine-tagged CeAhR LBD (S1,2). Each 10 µl sample was mixed 1 in 10 with load buffer prior to heating and running in each lane respectively. L is the ladder that consists of 5µl of suitable protein marker.

3.2 Expression of CeAhR LBD soluble protein component

After successful expression of CeAhR LBD in *E. coli*, experiments were carried out to determine if it is possible to obtain CeAhR LBD protein in a soluble form. Induction of CeAhR LBD by transforming BL21(DE3) was performed as described before. First, sonication of the protein samples was carried out, then centrifugation at 20,000xg, after which the supernatant was analysed. Both

pelleted and soluble samples were loaded on an SDS-PAGE gel. Figure 18 shows thick protein bands at about 29 kDa in the pellet samples (P1&2) and faint similar bands in the soluble samples (S1-4) showing that most of the expressed protein is insoluble.

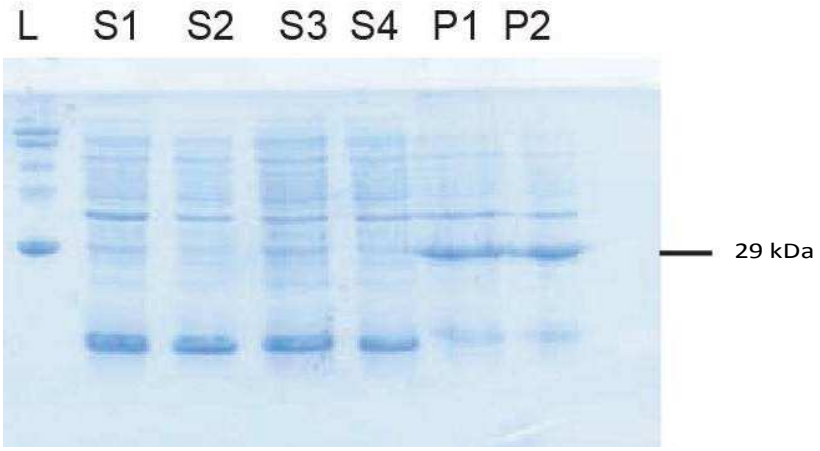


Figure 18. Solubility of AhR LBD in bacterial extracts; BL21(DE3) were transformed with pRSET plasmid containing sense CeAhR LBD, then induced with IPTG at 25°C or 30°C for 3 hours. Bacteria were lysed and the samples centrifuged at 20,000xg for ten minutes. The supernatant comprised soluble protein samples induced at 25°C (S1,3) or induced at 30°C (S2,4). The pelleted fractions (P1,2) were the non-soluble proteins induced at 25°C or 30°C respectively. Each 10 µl sample was mixed 1 in 10 with load buffer prior to heating and run in each lane as indicated. L is the ladder that consists of 5µl of suitable protein marker. SDS-PAGE was performed as described in the Materials and Methods.

Figure 18 shows that most of the expressed protein was insoluble. The soluble fraction was little. Lowering the temperature of expression produced more soluble protein as in sample S3. The soluble protein was separated from the pellet by ultra

filteration. Given that the 29kDa band in the soluble fraction was relatively faint, it was essential to confirm the identity of this protein. Its identity was confirmed by tryptic digest and sequencing of the protein band in the soluble sense lane. The results of this study showed that there is structural identity with CeAhR LBD in the sequence of 12 peptides, thereby proving that CeAhR LBD is present in the soluble fraction (Table 5).

Table 5. The amino acid sequence of the CeAhR LBD and highlighted in red (below) are 12 peptides sequenced from the protein bands following their tryptic digestion. Each peptide is identical to a portion of the CeAhR LBD.

MRGMRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPGFLRIDMRGKLMSLHGLPSSYVMGRTASGPVLG
HHHHHH TGGQQMGRDLYDDDDKDPGFLR

MICVCTPFVPPSTSDLASEDMILKTKHQLDGALVSMDQKVYEMLEIDETDLPMPLYNLVHVEDAVCMAE
HQLDGALVSMDQK

AHKEAIKNGSSGLLVYRLVSTKTRRTYFVQSSCRMFYKNSKPESIGLTHRLINEVEGTMLLEKRSTLKA
TYFVQSSCR NSKPESIGLTHRLINEVEGTMLLEK

KLLSFDDSFLQSPRNLQSTAALPL
LSFDDSFLQSPR

3.3 Expression of CeAhR LBD protein in Arctic Express cells at lower temperature

Arctic Express cells enable induction of target proteins at the lower temperature of 12°C, and additionally over-express a chaperone system; it was decided to determine whether these factors would enhance the expression of soluble CeAhR LBD. Arctic express cells were transformed and induced by IPTG using the same procedure as described before for BL21(DE3) cells. Figure 19 shows a thick band

at 29 kDa as the only difference between sense and anti-sense samples, demonstrating successful CeAhR LBD induction. The antisense band was found to be an endogenous protein of the same size as CeAhR LBD. Protein sequencing confirmed the presence of both CeAhR LBD and the endogenous protein. The protein bands can be seen in Figure 19.

Figure 19. SDS-PAGE gel for Arctic Express cells transformed with sense (S1-3) or antisense (A1-3) His-tagged CeAhR LBD and induced by IPTG at 12°C. Each 10 ml sample was mixed 1 in 10 with load buffer prior to sampling in each lane respectively. L is the ladder consisting of 1 in 6 protein marker. SDS-PAGE was performed as described in the Materials and Methods.

3.4 Comparison between expressed BL21(DE3) and Arctic Express transformed cells with histidine-tagged CeAhR LBD

Experiments were performed to compare and measure the amount of soluble 29kDa expressed protein after purification on a nickel column. The soluble components expressed by the cells, either BL21(DE3) or Arctic Express, were centrifuged at 250,000xg for thirty minutes to ensure that the samples contain soluble protein. Next, the soluble samples were purified by affinity chromatography on a nickel column. The eluted and wash samples of both BL21(DE3) and Arctic Express cells were compared to various amounts of BSA (1, 2, 4, 5 and 10 µg). The results revealed that both cells expressed less than 1µg of soluble CeAhR LBD protein in wash samples (Figure 20). Furthermore, the Arctic Express results (W1, W2) were better than those of BL21(DE3) cells (W). It was possible to calculate the amount of soluble protein from a known volume of culture. W1 and W2 are 2% of the soluble CeAhR LBD from 450 ml of bacterial culture; thus estimating a yield of ~0.1 mg per litre of culture. Therefore, the amount of CeAhR LBD is approximately 1 mg per 10 litres of culture, which is inadequate for crystallization experiments.

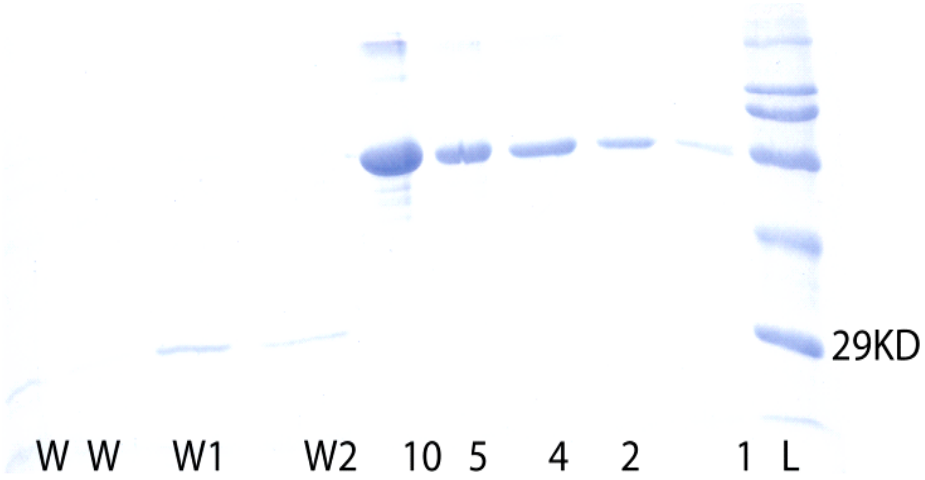


Figure 20. SDS-PAGE gel of affinity-purified samples. CeAhR LBD was induced in BL21(DE3) (W) or Arctic Express (W1, W2) cells and the soluble fraction isolated by sequential centrifugation at 2000xg for 10 minutes and 250,000xg for 30 minutes. Samples were then purified on a nickel affinity column and eluted samples are shown. 1, 2, 4, 5 and 10 are BSA samples of 1, 2, 4, 5 and 10 µg. L is ladder consisting of 3 µg of 1 in 6 protein marker. Each sample was loaded with 1 mg protein loading dye before sampling in each lane. SDS-PAGE was performed as described in the Materials and Methods.

3.5 Expression of CeAhR LBD in *C. elegans*

The expression of CeAhR LBD in bacteria was successful but most of the expressed protein was insoluble and unsuitable for crystal structure work. We need a large volume (20 litres) of culture to purify just 1 mg of soluble protein. The next step was to try and express CeAhR LBD in higher eukaryotic system. The *C. elegans* itself was chosen to express its own protein hoping that it has all the chaperones needed to fold CeAhR LBD.

Three constructs were prepared for expression of CeAhR LBD in *C. elegans*, namely pPD30.38 CeAhR LBD His tag, pPD30.38 CeAhR LBD GST and pPD30.38 GST (negative control). Microinjection of these constructs was performed by Declan Brady. The constructs were injected with M cherry as a fluorescent marker indicating successful microinjection and creation of transgenic animals. Good expression of the control GST pPD30.38 was obtained; however, the expression of both CeAhR LBD constructs was negative with good expression of the control M Cherry. The protein production was checked by western blot, but no protein band of appropriate size was found, indicating failure of the expression system. The transgenic animals' genomic DNA was extracted, and the existence of the constructs confirmed. The transgenic CeAhR LBD carrying animals were lysed and the genomic DNA was extracted. Suitable primers (primers 385 forward and reverse) were used to amplify the CeAhR LBD from the genomic DNA extracted from the transgenic C. *elegans*. The amplified DNA was run on 1% agarose gel and both His and GST tagged CeAhR LBD were confirmed before starting protein expression to avoid false negative results.

3.6 Expression of histidine tagged CeAhR LBD in transgenic *C. elegans*

After successful cloning of CeAhR LBD in transgenic *C. elegans*, experiments were performed to determine if it was possible to obtain CeAhR LBD protein, especially in soluble form. The protein samples were sonicated and centrifuged at 500xg. Samples were loaded on an SDS-PAGE gel. The results showed no

significant CeAhR LBD band at the expected 29 kDa, suggesting no or weak expression (Figure 21).

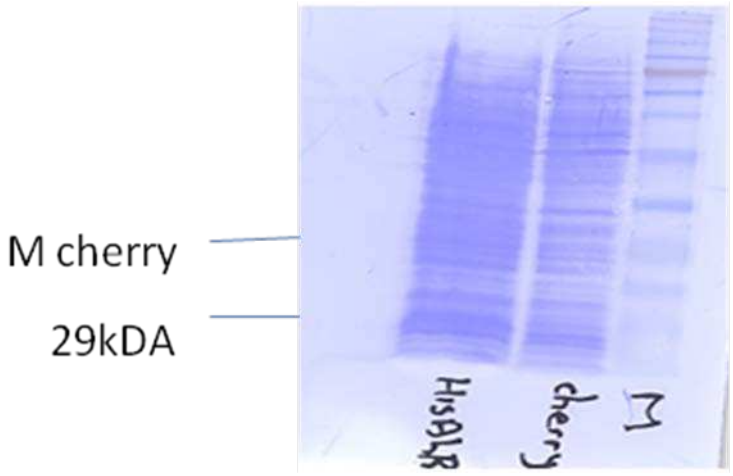


Figure 21. Commassie-stained protein gel of CeAhR LBD protein extracted from transgenic *C. elegans*. The animals were microinjected with His-tagged CeAhR LBD (left lane) or with M cherry (middle lane labelled "cherry"). M is protein marker.

The samples showed expressed proteins from both M cherry and CeAhR LBD microinjected animals. There were no expression bands different from the control for CeAhR LBD samples. These results indicated weak or no expression of CeAhR LBD in transgenic animals. The expression of M cherry was fine. To compare both samples to each other, the M cherry band does not exsist in the CeAhR LBD sample and the CeAhR LBD band does not appear in the M cherry sample. By estimating the expected bands from the size marker, it could be possible to confirm the expression of the M cherry but there was no evidence for His-tagged CeAhR LBD. Co microinjection of M cherry and CeAhR LBD aimed

to create transgenic animals expressing CeAhR LBD; M cherry gave red florescence that helped selection of the transgenic strains for expression.

3.7 Expression of GST tagged CeAhR LBD in transgenic *C. elegans*

GST CeAhR LBD was successfully microinjected in *C. elegans* and the transgenic animals were crushed to detect CeAhR LBD protein. The experiment was carried out with pPD30.38 GST as a positive control indicating the integrity of the system. Normal *C. elegans* animals were crushed and their protein used as negative control. The experiment showed a clear GST band (Figure 22), but unfortunately, no GST CeAhR LBD protein band was detected raising questions regarding the practicality of using this system for expression of CeAhR LBD.

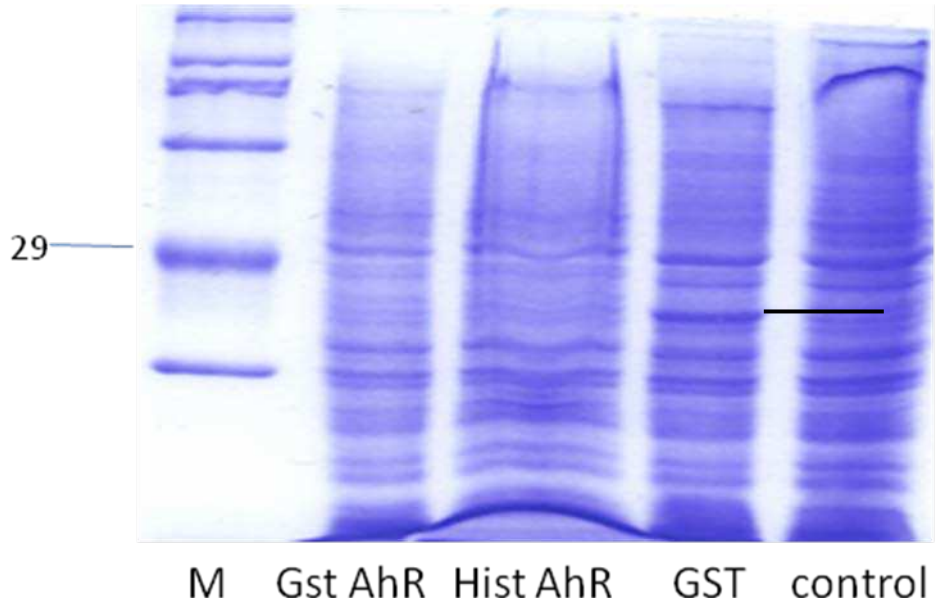


Figure 22. Protein gel stained with Commassie stain. M is protein molecular weight marker. GST AhR is the GST tagged CeAhR LBD sample. Hist AhR is the His tagged CeAhR LBD sample. GST is the sample with the expressed GST tag only. The negative control is uninjected *C. elegans*. The black line shows the GST band (of 35 kDa in the GST-pPD30.38 lane that is not in the negative control lane indicating succesful expression of GST) as a control indicating transgenic *C. elegans* working as expression system.

Figure 22 shows proteins expressed from both GST CeAhR LBD and His tagged CeAhR LBD constructs. The experimental design included a negative control, which comprises the protein extracts from uninjected *C. elegans*. On the other hand, *C. elegans* carrying GST pPD30.38 is another control that proves the integrity of the transgenic *C. elegans* worms in expressing recombinant foreign protein. From Figure 22, the GST band was seen in contrast with the negative control sample. Yet, no recombinant CeAhR LBD bands at 29kDa (His-tagged

CeAhR LBD) or 54 kDa (GST-tagged CeAhR LBD) were detected. The system was not expressing CeAhR LBD or expresses it at a very low level. The following step was to perform western blot, which could detect protein bands down to 1 ng.

3.8 Western blot of expression of CeAhR LBD

Commassie stained protein gel can detect down to 100 ng protein bands. To evaluate the expression of CeAhR LBD at a lower scale, a western blot was performed on the gel samples run by electrophoresis. Figure 23 shows His tagged CeAhR LBD expression for comparison with negative control. From the gel, it is difficult to detect the clear band of CeAhR LBD protein. The western blot was repeated many times, yet the possible CeAhR LBD band was inconsistent; the expression trials were repeated 4 times with similar results. In Figure 23, a band can be seen in the CeAhR LBD lane that is different from the control. However, the antibodies were not specific enough to detect the His tag only. It gave results similar to the protein gel stained with Commassie, and again it was difficult to confirm expression of CeAhR LBD. The system was not practical for expressing a large enough amount of protein for crystal structure work.

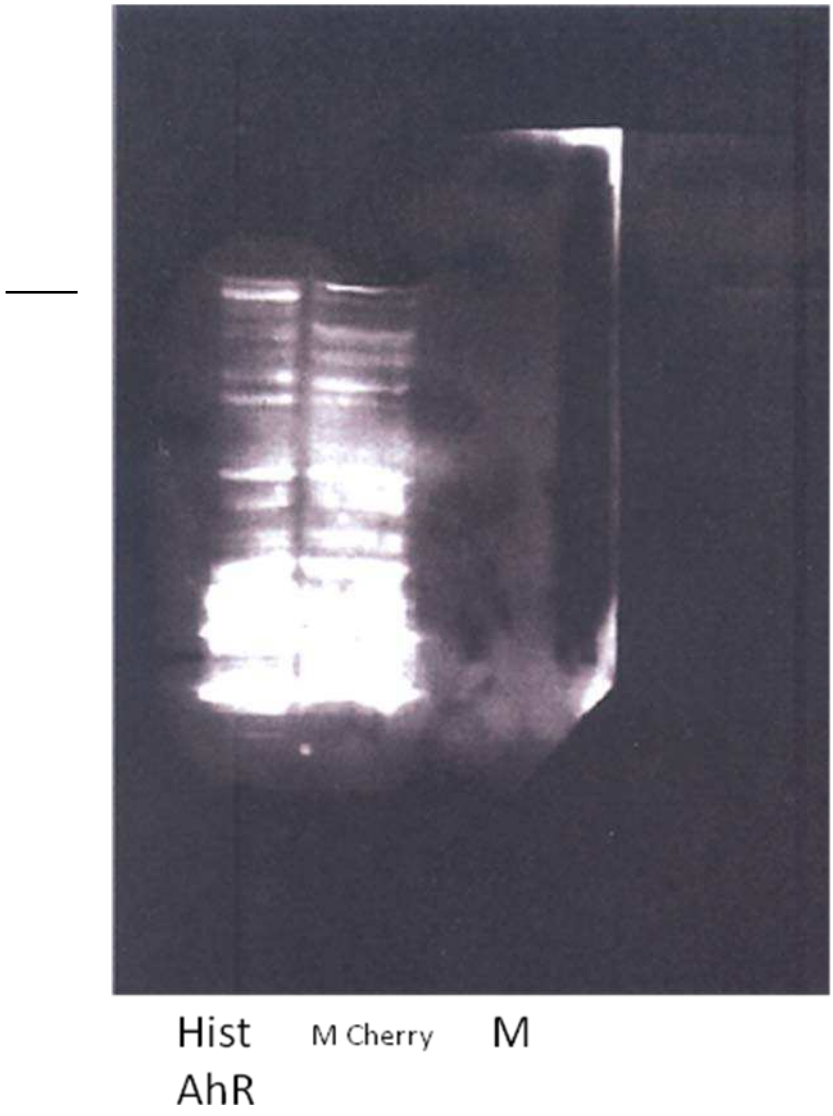


Figure 23. Western blot of proteins extracted from transgenic *C. elegans* carrying His tagged CeAhR LBD constructs on the left. The lane lying in the middle showed the expressed protein of transgenic *C. elegans* microinjected with M cherry. The lane on the right side is that of the marker. The black line indicates the possible expressed CeAhR LBD band.

The western blot of CeAhR LBD samples in Figure 23 showed multiple bands binding to the anti histidine antibodies. These results could be explained by excess

antibody loading or by less specificity against transgenic *C. elegans* proteins. The marker did not appear. By comparison between both CeAhR LBD and M cherry, nearly no bands expressing CeAhR LBD were detected. Unfortunately transgenic CeAhR animals were not suitable for the production of soluble CeAhR LBD for crystal structure study.

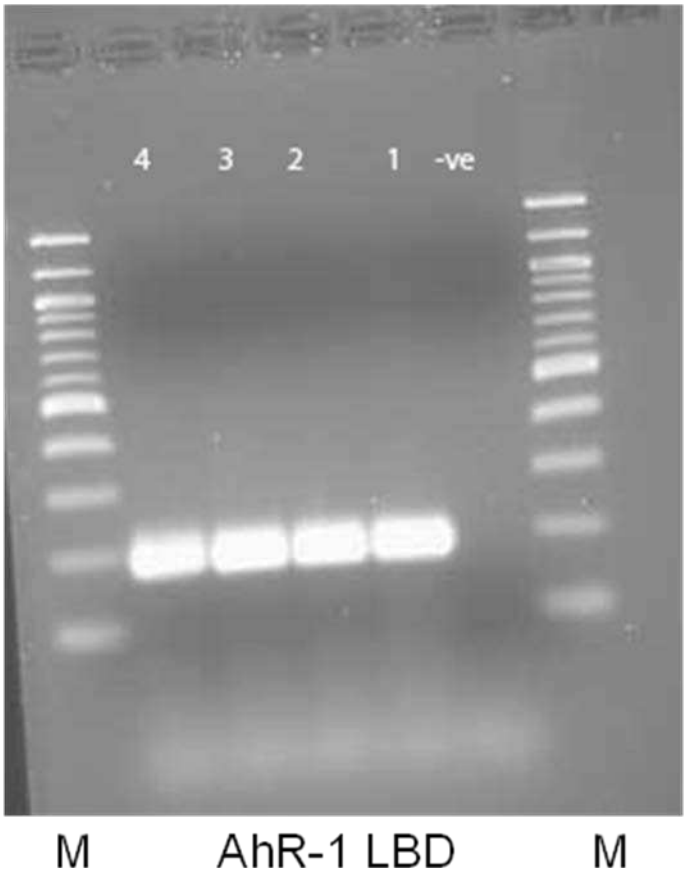


Figure 24. Agarose gel showing amplified CeAhR LBD from pPD30.38 constructs by PCR reaction. –ve indicates the negative control sample containing the PCR master mix and the primers, but without DNA to exclude contamination. Samples 1, 2, 3, 4 are four PCR products of CeAhR LBD with new restriction sites XbaI and SacI with different TM of the reaction of 54, 56, 58 or 60°C respectively.

To show that transgenic CeAhR animals are not suitable for CeAhR LBD expression, DNA amplification was performed to confirm the presence of CeAhR LBD constructs inside the transgenic animal. This step was important to avoid bias in the results of the expression system. The samples in Figure 24 show successful integration of CeAhR LBD DNA into the genome of transgenic *C. elegans* strains.

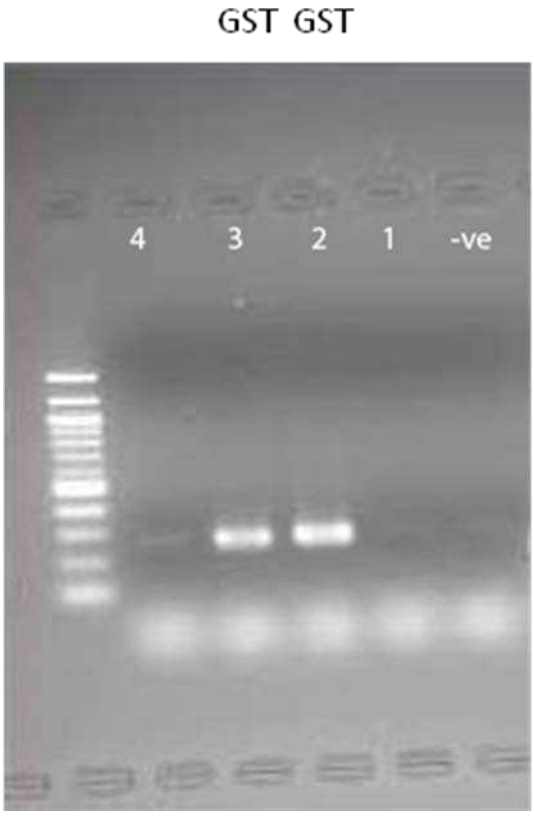


Figure 25. 1% agarose gel carrying PCR products of amplified GST pPD30.38 construct integrated in the genome of transgenic *C. elegans* after microinjection. –ve represents the negative control sample for the PCR reaction containing no DNA. Samples 2 and 3 are DNA extracted from GST-expressing transgenic *C. elegans*. Samples 1 and 4 are GST pPD30.38 with different TM.

The experiment illustrated in Figure 25 was performed to confirm the integration of pPD30.38 constructs into the *C. elegans*' genome so as to avoid false negative results. The DNA was amplified by Taq polymerase that was used to amplify the CeAhR LBD.

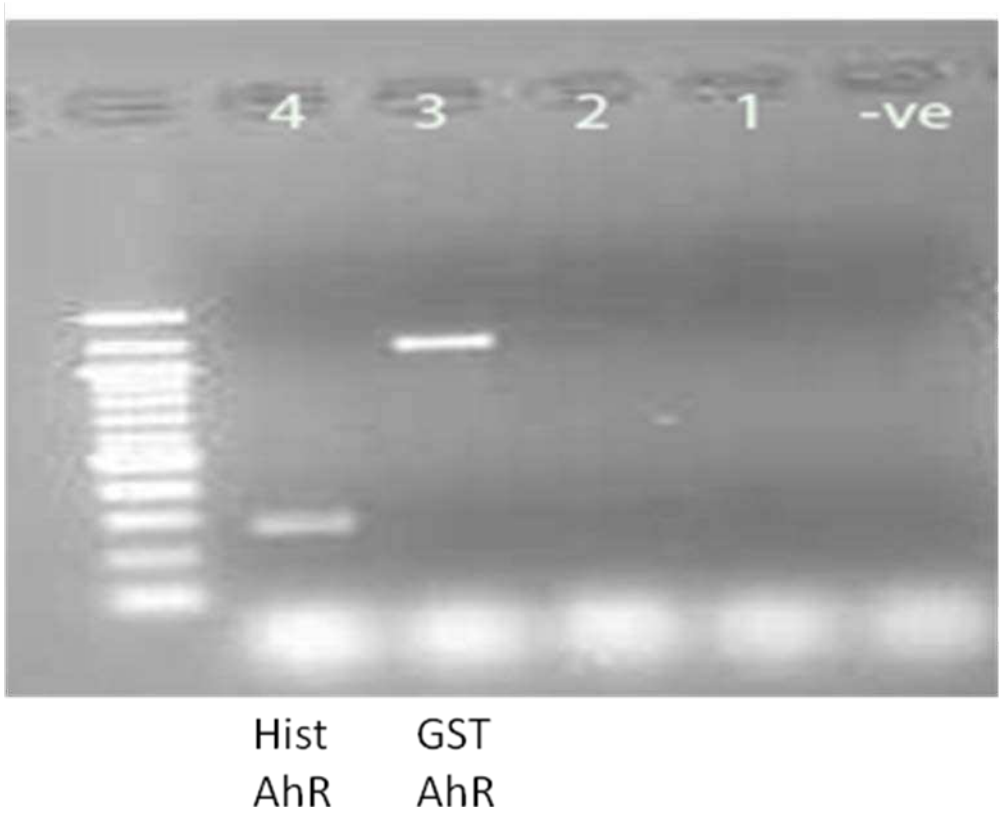


Figure 26. 1% agarose gel carrying DNA marker on the left. The negative (–ve) control was a sample carrying no DNA. Sample 1 carried histidine tagged CeAhR LBD construct microinjected in C. elegans. Sample 2 carried GST tagged CeAhR LBD construct microinjected in C. elegans. All samples were amplified PCR products of the mentioned constructs.

Another confirmatory step of successful microinjection of CeAhR LBD into *C. elegans* was performed. The difference was the new primers used to amplify the whole CeAhR LBD construct, not just part of it, to add more data about successful integration of the constructs inside the transgenic animals' genome. The gel (Figure 26) shows DNA marker on the left. Hist AhR represents the sample

carrying amplified DNA from His tagged CeAhR LBD, while GST AhR is the GST tagged CeAhR LBD. The CeAhR LBD has two tags, His tag and GST tag, generating fragment sizes of 600 and 1.5 kb respectively. The sizes of these fragments were equal to the two fragments seen in the gel, suggesting successful integration of both tagged AhR constructs into the genomic DNA of *C. elegans*. The experiment was done to avoid false negative results. The transgenic animals may mutate and lose the pPD30.38 integrated plasmid carrying the target construct. The expression of CeAhR LBD protein was not abundant enough. It is important to check the genomic DNA for successful integration of the target recombinant DNA to avoid bias. From the above gel, it can be said that the cloning process was successful, however, the expression system (*C. elegans*) was not capable of expressing a large amount of recombinant CeAhR LBD in the muscle wall using the UNC 54 promoter.

3.9 Expression of CeAhR in yeast system

The third system of expression was *Pichia pastoris* GS 115 Mut+. Figure 27 simplifies the 4 recombinant proteins expected to be expressed in this system. pPICZ alpha B contains alpha factor that helps secretion of CeAhR LBD protein, either with His tag or GST tag. On the other hand, pPICZ B constructs are expected to express CeAhR LBD in the intracellular compartment. C-myc is an epitope expressed by pPICZ alpha B and pPICZ B. The Pichia system has many advantages over bacterial ones. It is eukaryotic system that has post translational

modification capacity. Also, it is possible to express a large biomass in small volumes of liquid media.

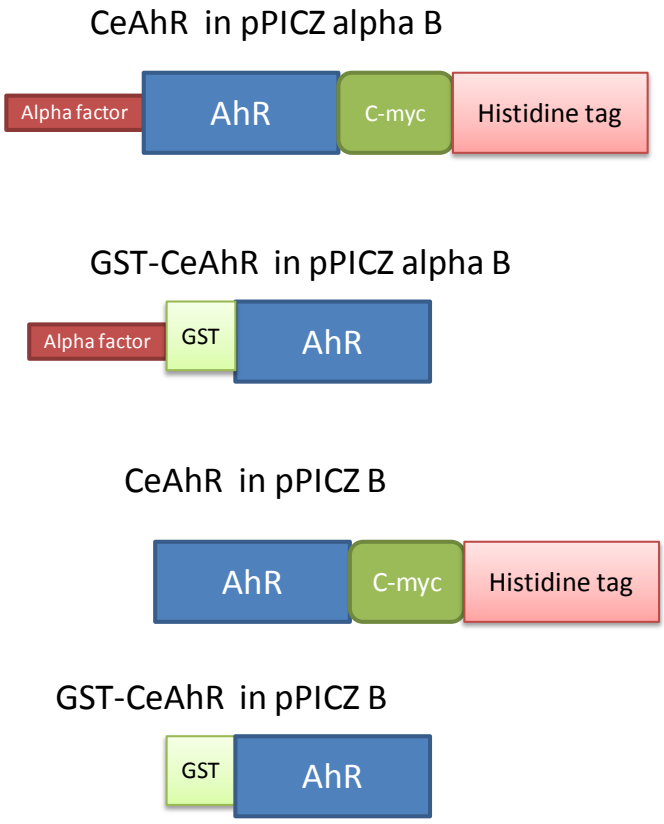


Figure 27. Four different CeAhR LBD proteins generated by the 4 plasmid constructs transformed in *Pichia pastoris*.

3.10 Cloning of CeAhR LBD

Experiments were carried out to clone PCR-amplified CeAhR LBD, or cut CeAhR LBD fragments, from Prset or PET41b plasmids. The PCR-amplified CeAhR LBD

with EcoR1 and Xba1 sites was cloned into the plasmid pGEMT. The new construct was transformed into JM109. To evaluate the transformation success, the transformed cells were plated on LB agar/ampicillin/IPTG/Blue-Gal plates. The resistance to ampicillin was expected to select the plasmid containing the plasmid pGEMT. There were no colonies in the negative control, as JM109 without plasmids cannot grow on ampicillin. The positive controls contain plasmids with an antibiotic-resistant gene. A known amount of antibiotic resistant gene acted as a transformation control. Each 1µg of DNA should yield $1x10^8$ colonies. In theory, all positive controls or sample controls should produce colonies that are white in colour. The white colonies indicate successful integration of the recombinant gene in the plasmid (pGEMT). The results of cloning of CeAhR LBD in pGEMT plasmid are summarized in Table 6. It can be noticed the more insert /vector ratio, the more successful cloning results detected.

Table 6. Colony growth outcomes from transformation of JM109 with CeAhR LBD. The negative control had no plasmid added, the control insert is a positive control with ligated control insert transformed into JM109 cells via ampicillin-resistant plasmid, and background control was re-ligated with double digested vector (adding ligase to the double digested DNA). The standard reaction is the ligation reaction with DNA (CeAhR LBD constructs) transformed into JM109 by ligation into pGEMT plasmid.

Plate type	Number of colonies
Negative control	0
Control insert	220 (60% white)
Background control	20
Standard reaction Plate1 - insert:vector ratio 1:1 Plate2 - insert:vector ratio 3:1 Plate3 - insert:vector ratio 10:1	 35 100 200

After successful transformation, the CeAhR LBD pGEMT construct was double digested by Ecor1 and Xba1 restriction digestion enzymes, and run on 1% agarose gel. The DNA lane showed two bands, the larger one was about 3000 bp and the smaller about 600 bp, which was supposed to be CeAhR LBD. For the AhR GST pGEMT construct, the smaller fragment was 1500 bp (Data not shown). The CeAhR LBD band was gel purified and both pPICZ B and pPICZ alpha B were double digested with both EcorI and XbaI. The vectors CeAhR LBD and CeAhR

LBD-GST were ligated using the same method applied before for sub-cloning CeAhR LBD in pGEMT. The results are given in Table 7.

Table 7 shows transformation of CeAhR LBD into pPICZ alphaB. The CeAhR LBD was double digested from pGEMT vector with EcoRI and XbaI and cloned in pPICZ alpha B insert that was opened with same restriction digestion enzymes.

Plate type	Number of colonies
Transformation control	100 → transformation efficiency was $1x10^8$ per µg DNA
Background control	25
Negative control	0
Standard reaction	250

Table 8. CeAhR LBD with BamHI restriction digestion site

GGATCCTGGATTTTTGAGAATTGACATGCGCGGAAAGTTGATGTCTCT
ACATGGATTACCATCATCGTATGTAATGGGAAGAACTGCCTCGGGTCC
AGTGCTCGGAATGATTTGCGTTTGCACACCTTTTGTGCCGCCTTCAAC
ATCCGATTTAGCATCCGAAGACATGATTTTGAAAACAAAACATCAGTT
GGATGGAGCTTTAGTATCTATGGATCAAAAGGTTTATGAAATGTTAGA
AATTGATGAAACTGATCTACCAATGCCACTCTATAATCTAGTCCACGT
GGAAGATGCAGTCTGCATGGCTGAAGCTCATAAAGAAGCTATCAAAA
ACGGGTCATCTGGTCTTCTGGTATATCGTCTAGTCAGCACAAAAACTC
GTCGTACGTATTTTGTTCAAAGCTCCTGTAGGATGTTTTACAAGAATA
GCAAACCGGAATCAATTGGCTTAACTCACAGATTACTCAACGAAGTG
GAAGGTACAATGCTTTTAGAAAAAAGAAGCACATTGAAAGCTAAACT
ATTATCATTTGACGATTCATTTCTTCAATCTCCACGAAATCTCCAATCA
ACAGCTGCACTTCCATTATAAGGATCC

The CeAhR LBD with both BamHI sites was used for its expression in pRSET vector. The plasmid adds a polyhistidine tag to the CeAhR LBD protein to aid the purification process.

Table 9 CeAhR LBD with EcoRI and XbaI sites

GAATTCACATGGGATTTTTGAGAATTGACATGCGCGGAAAGTTGATG
TCTCTACATGGATTACCATCATCGTATGTAATGGGAAGAACTGCCTCG
GGTCCAGTGCTCGGAATGATTTGCGTTTGCACACCTTTTGTGCCGCC
TTCAACATCCGATTTAGCATCCGAAGACATGATTTTGAAAACAAAAC
ATCAGTTGGATGGAGCTTTAGTATCTATGGATCAAAAGGTTTATGAAA
TGTTAGAAATTGATGAAACTGATCTACCAATGCCACTCTATAATCTAG
TCCACGTGGAAGATGCAGTCTGCATGGCTGAAGCTCATAAAGAAGC
TATCAAAAACGGGTCATCTGGTCTTCTGGTATATCGTCTAGTCAGCAC
AAAAACTCGTCGTACGTATTTTGTTCAAAGCTCCTGTAGGATGTTTTA
CAAGAATAGCAAACCGGAATCAATTGGCTTAACTCACAGATTACTCAA
CGAAGTGGAAGGTACAATGCTTTTAGAAAAAAGAAGCACATTGAAAG
CTAAACTATTATCATTTGACGATTCATTTCTTCAATCTCCACGAAATCTC
CAATCAACAGCTGCACTTCCATTATTTCTAGA

For expression of CeAhR LBD in *Pichia pastoris*, the CeAhR were amplified by PCR with the new restriction digestion sites, EcorI and XbaI, as shown in Table 9, to suit cloning in pPICZ B and pPICZ alpha B vectors.

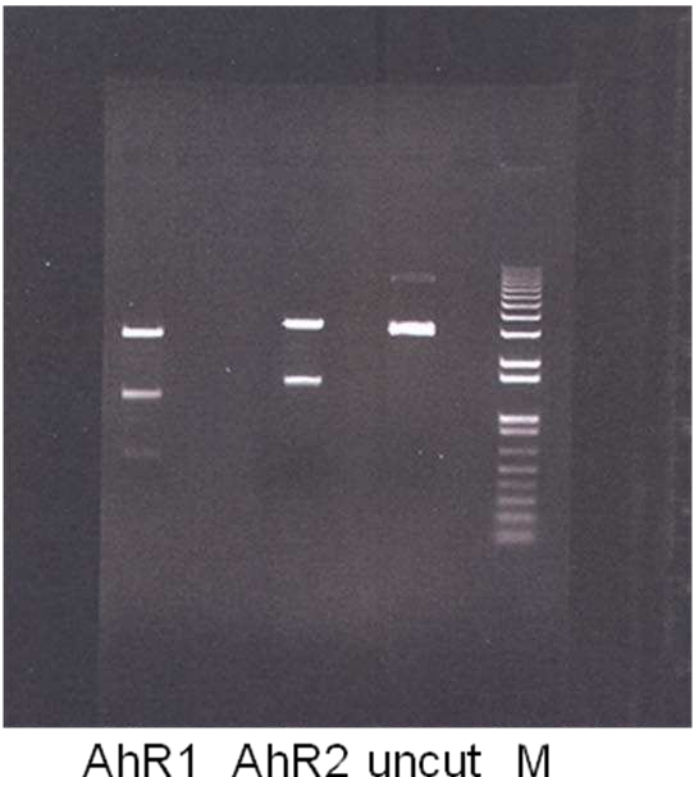


Figure 28. 1% agarose gel carrying CeAhR LBD GST construct in pPICZ alpha B. M is DNA marker. Uncut indicates the control using uncut CeAhR LBD GST in pPICZ alpha B. AhR2 is the double digested CeAhR LBD with EcorI and XbaI. The 1.5 kb band in AhR2 lane is suspected to be the GST-tagged CeAhR LBD fragment. AhR1 is AhR GST supposed band cut with BamHI that gave 3 bands; the middle one is supposed to be AhR band in AhR1 lane (1100kb).

After successful cloning and transformation, the colonies were picked up and mini-prepped to extract clean plasmid DNA that is digested with suitable restriction digestion enzymes. Agarose gel was run and the size of the expected

CeAhR LBD was detected indicating the right cloning (for example Figures 28, 29 and 30). The final step was to sequence the plasmid and make sure no errors happened in the construct especially in the place where the construct starts to bind the new vector. This strategy was applied to all 4 constructs before attempting expression.

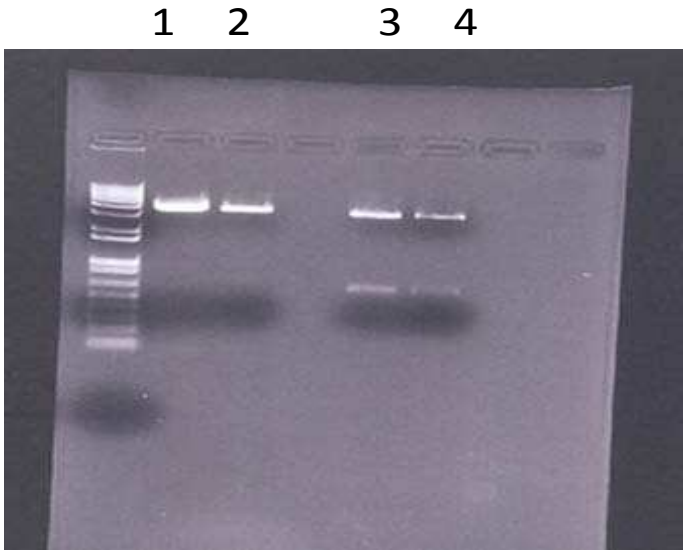


Figure 29. 1% agarose gel of His tagged CeAhR LBD construct in pPICZ B. It is double digested with Ecor1 and Xba1. The cut fragment showed the expected 600 bp in (S3,S4) of His-tagged CeAhR LBD cloned in pPICZ B in samples 3 and 4. The uncut samples were used as control in samples 1 and 2.

The CeAhR LBD fragments were cut with Ecor1 and Xb1 restriction digestion enzymes (Figure 29). 600 bp fragments can be seen in samples 3 and 4. These two fragments are supposed to be CeAhR LBD. The plasmid construct was confirmed by sequencing.

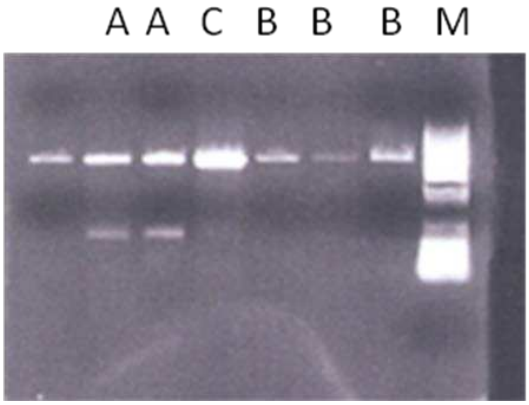


Figure 30. 1% agarose gel carrying M which is DNA marker. B is pGEMT without AhR constructs. C is uncut pGEMT construct. A is pGEMT carrying His tagged CeAhR LBD.

The CeAhR LBD fragments were cut with Ecor1 and Xba1 restriction digestion enzymes (Figure 30) as previously (Figure 29). 600 bp fragments can be seen in lanes labelled A. These two fragments are supposed to be CeAhR LBD. The plasmid construct was confirmed by sequencing.

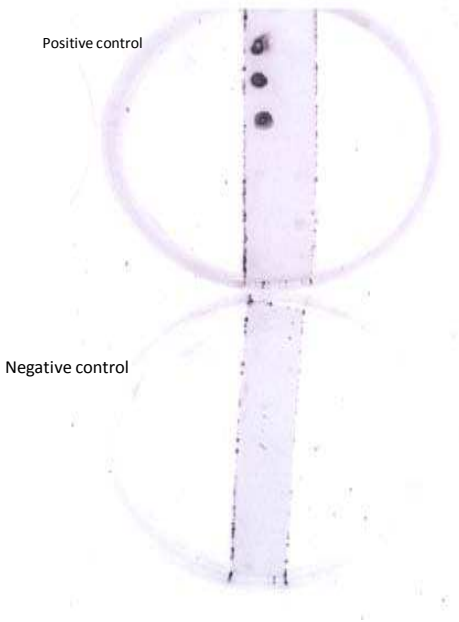


Figure 31. Nitrocellulose membrane showing dot blotting of positive control His tag containing protein in the upper membrane strip with serial dilution of protein concentration, and negative sample in the lower nitro cellulose membrane strip. The negative control sample included bacterial and yeast extract proteins.

To screen for the best colony expressing CeAhR LBD, 100 colonies were examined by dot blotting against the histidine and GST tags of expected expressed CeAhR LBD. The antibodies were subjected to test the specificity and the sensitivity against bacterial and yeast protein before starting the blotting experiment. A dot blotting experiment was done to evaluate the specificity and the sensitivity of the Anti His tag antibodies (Figure 31). The results showed that the

antibodies were sensitive to bacterial or yeast extract proteins. It is sensitive to ng protein concentrations.

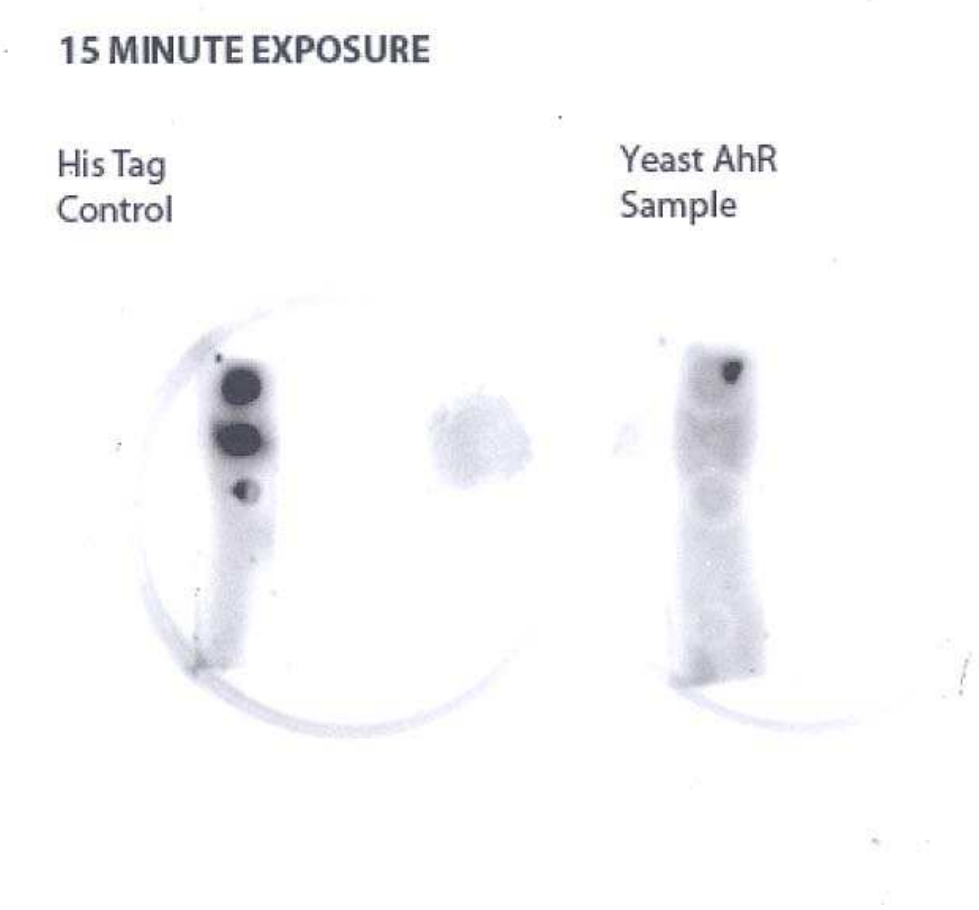


Figure 32. Dot blotting of secreted protein from a His-tagged CeAhR LBD transformed colony on the right nitrocellulose strip. Control nitrocellulose membrane carrying both positive and a negative controls are on either ends of the left strip. The strip was incubated with anti His antibodies. The ECL kit (western blot labiling kit) was added. The membrane was photographed by the BIO-RAD gel doc machine.

A dot blot experiment showed that one transformed colony was positive with His-tag antibodies (Figure 32). This promising result is not enough because dot blot is

just a screening method. It is supposed to screen 100 transformed CeAhR LBD secreting colonies to detect the best one that could be reliable for large scale expression. The following step was to confirm the results with western blotting that indicate the most reliable results.

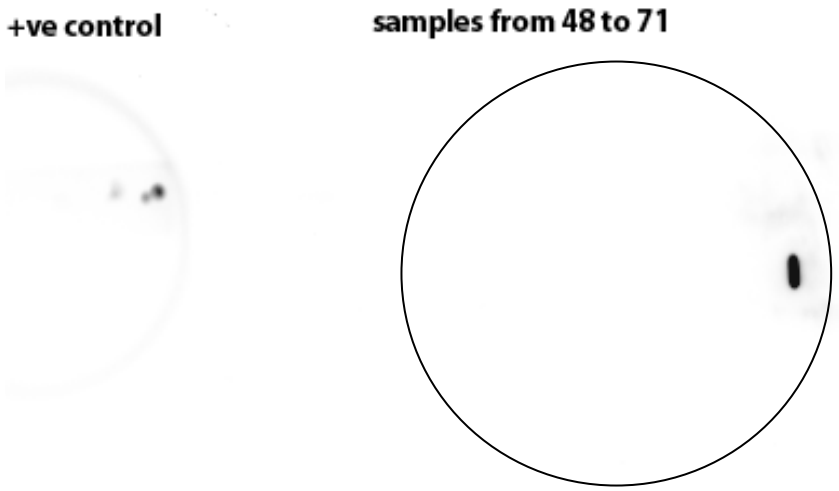


Figure 33. Screening of different dot blotted CeAhR LBD with His tag. 1ml secretions of 100 colonies were blotted on nitrocellulose membrane and incubated with anti His tag antibodies. ECL kit (western blot labeling kit) was added and the membranes were photographed by the gel doc machine. All results were negative except one colony, No. 71. The left plate is the control plate. The right plate contains 24 dotted samples.

The dot blot screening of CeAhR LBD secreting colonies with His-tag showed that no colony managed to secrete the protein except one potential one which was colony No. 71.

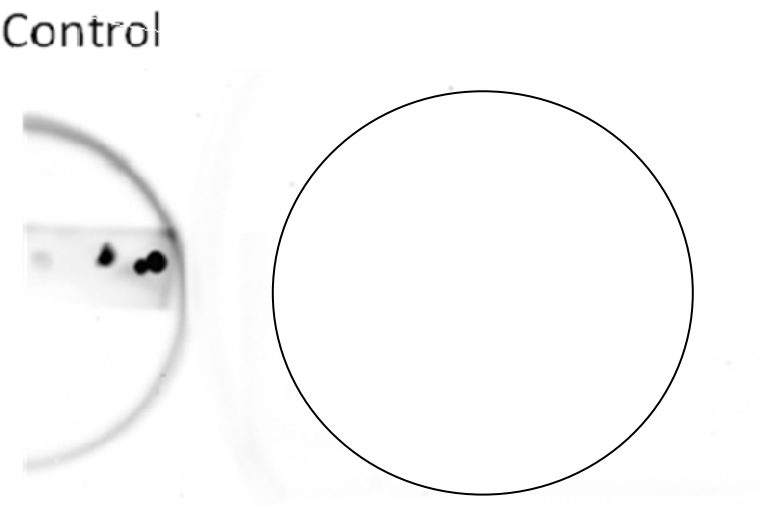


Figure 34. Screening of different dot blotted CeAhR LBD with GST tag. 1ml secretions of 100 colonies were blotted on nitrocellulose membrane and were incubated with anti GST tag antibodies. ECL kit was added and the membranes were photographed by the gel doc machine. All results were negative. The samples on the left are positive control samples.

The screening of GST-AhR secreting colonies showed that no colony could secret any CeAhR LBD protein. From both Figures 33 and 34 it was supposed that CeAhR LBD was poorly or not secreted from *Pichia pastoris* transformed colonies. After screening 200 colonies, only one colony gave a potentially positive result. The samples could be tested by staining a gel with SYPRO RUBI that could detect down to 1ng protein, as in Figures 35, 36 and 37.

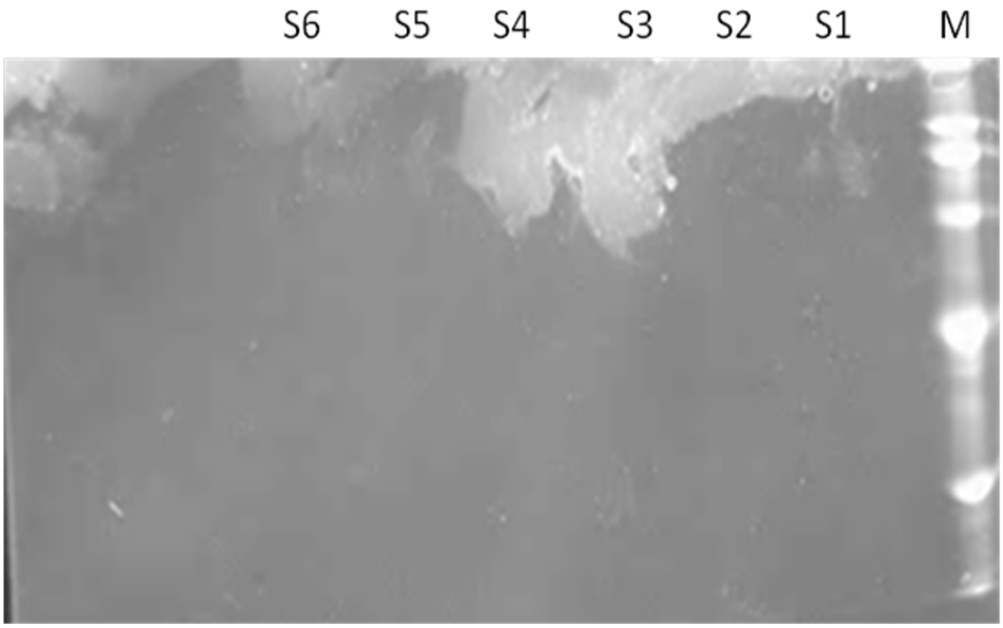


Figure 35. Protein gel stained with SYPRO Rubi stain. S1-6 is secreted His-tagged CeAhR LBD expression from yeast extract.

A gel of proteins extracted from His-tagged CeAhR LBD tansformed colonies (Figure 35; S1, S2, S3, S4, S5 and S6), showed that the yeast extract contained no detected CeAhR LBD protein. From this experiment, there is weak evidence that CeAhR LBD is secreted by the *Pichia pastoris* system.

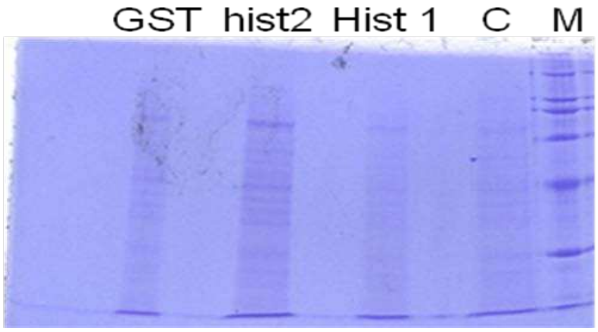


Figure 36. Protein gel stained with Commassie. C is control Pichia (without any constructs) protein extract after cell lysis, Hist1 is His-tagged CeAhR LBD secreting Pichia, Hist2 is intracellular expressing His-tagged CeAhR LBD Pichia, GST is intracellular GST CeAhR LBD expressing Pichia.

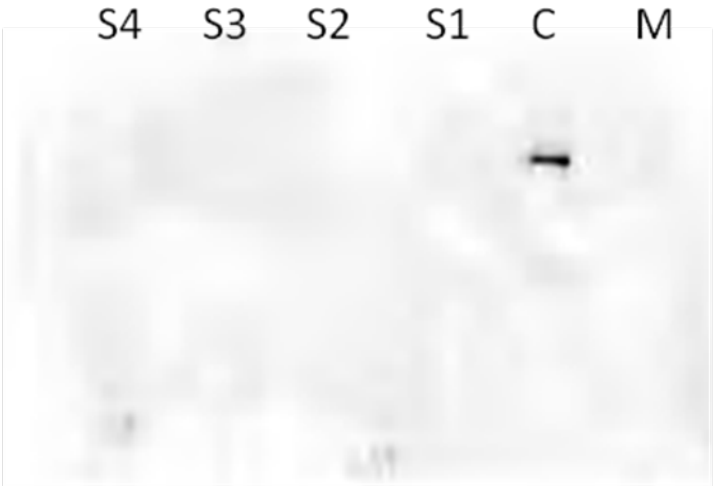


Figure 37. Western blot of proteins extracted from Pichia. M is marker protein. C is Pichia extracted proteins without any constructs as negative control. S1,2 are secreted His-tagged CeAhR LBD samples. S3,4 are intracellular His-tagged CeAhR LBD expression samples.

This experiment in Figure 37 indicated that the *Pichia pastoris* expression system failed to manufacture CeAhR LBD, neither secreting it nor producing it

intracellularly. The western blot outcome for GST CeAhR LBD was the same. Therefore, *Pichia pastoris* is not a suitable system to produce CeAhR LBD. The positive transformed colony from the screening was streaked onto a fresh plate and a new colony was picked up and grown and proteins secreted in the media were western blotted to detect any CeAhR LBD protein in S1 sample. Another fresh colony was transformed and the suspected CeAhR LBD was blotted to confirm if any protein can be detected. At the same time CeAhR LBD transformed colonies were grown and a trial to express CeAhR LBD in the intra cellular compartment was attempted. The cells were lysed and the desired protein was detected by a western blot of two samples from different colonies. The western blot was totally negative to dectect either secreted or internal CeAhR LBD. So it could be said that *Pichia pastoris* was not suitable for CeAhR LBD expression.

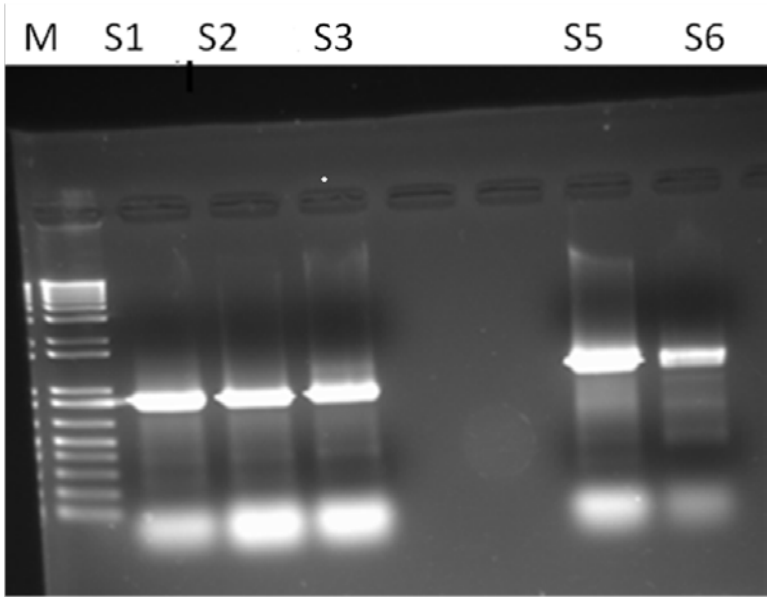


Figure 38. 1% agarose gel of amplified genomic DNA from *Pichia pastoris* carrying His-tagged CeAhR LBD in pPICZ alpha B control (S1), His-tagged CeAhR LBD from pPICZ B construct extracted from genomic DNA (S2 and S3), GST-tagged CeAhR LBD in pPICZ alpha B control (S5) or GST-tagged CeAhR LBD in pPICZ alpha B extracted from genomic DNA (S6). The PCR product bands were amplified by AOX1 primers.

The expression of CeAhR LBD resulted into poor out come. As applied in transgenic *C. elegans* the genomic DNA was amplified to make sure that the CeAhR LBD constructs were integrated in the genome of the yeast. This experiment shown in Figure 38 indicates successful integration of CeAhR LBD constructs in the *Pichia pastoris* genome. The genomic DNA was extracted from the transformed yeast samples. The control samples were pure plasmids carrying the constructs. Successful integration of the construct in the genome was confirmed by amplified DNA by PCR reaction from both the control and the

genomic DNA giving two equal bands amplified from both genomic and pure plasmid template DNA.

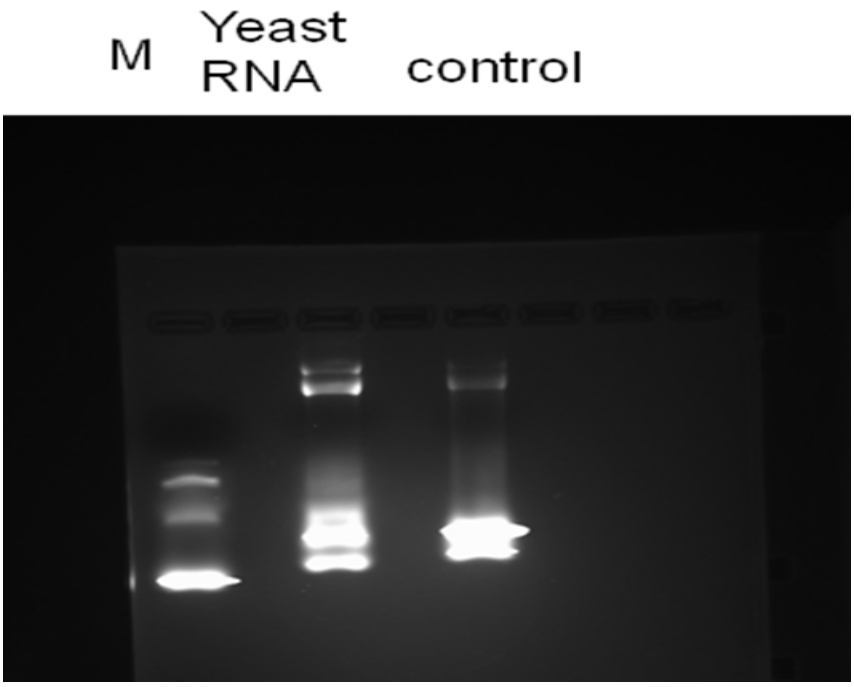


Figure 39. 1% agarose gel with 1% SDS-PAGE. The samples marked M have DNA marker. The Yeast RNA sample was total RNA purified from CeAhR LBD expressing yeast. Control indicates already intact purified rat total RNA extracted from the liver.

An experiment was then conducted to check that the extracted RNA was intact and ready for reverse transcriptase PCR (see below). The RNA sample was not smeared indicating little or no degradation of the yeast RNA. The samples in Figure 39 showed intact total RNA extracted from *Pichia pastoris* expressing His-tagged CeAhR LBD. There was no smearing, and the amount of RNA was abundant.

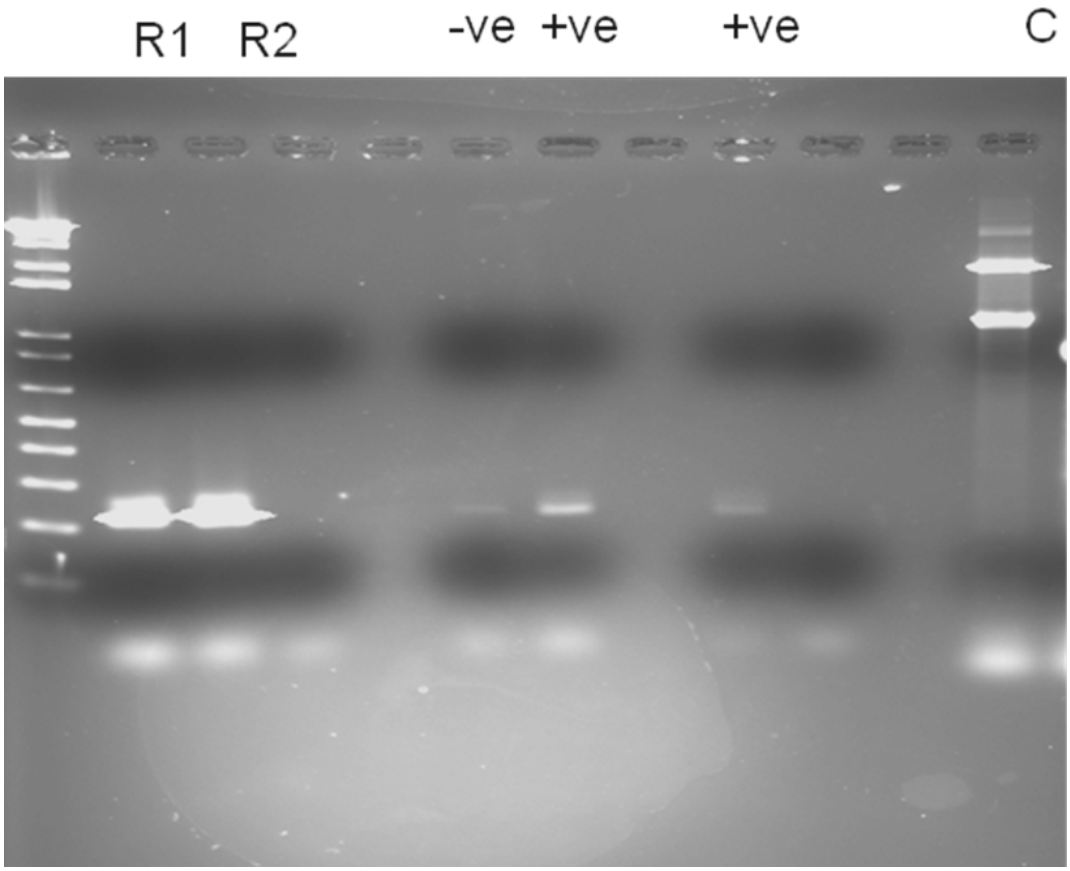


Figure 40. Reverse transcriptase (RT) PCR of CeAhR LBD cDNA. The figure shows 1% agarose gel for RT PCR of cDNA made from total RNA extracted from *Pichia pastoris*. The samples consist of DNA marker in the extreme left (KB+). R1,R2 are amplified DNA by PCR from cDNA made from yeast extracted total RNA. –ve control sample is RNA amplified by PCR to evaluate genomic contamination. +ve control samples are template CeAhR LBD construct in pPICZ B. These DNA samples were amplified with specific CeAhR LBD primers. C is PCR (using AOX 1 primers) of *Pichia pastoris* CS 115 transformed with pPICZ B, which confirms the presence of endogenous alcohol oxidase, that is to say that this is *Pichia pastoris* Mut+ strain.

The experiment illustrated in Figure 40 indicates the presence of the RNA transcript from the CeAhR LBD gene integrated in the genome of *Pichia pastoris*.

3.11 Effects of AhR agonists on the viability of *C. elegans*

Six compounds were tested for their effects on *C. elegans*, namely TCDD, AZ1c, PCB126, AMB, 3MC and β-naphthoflavone. 1 M sodium azide was used as positive control and 1 mM DMSO as vehicle control. *C. elegans* N2 animals without any toxin were used as a negative control. The results showed that sodium azide killed all animals, indicating a successful positive control. No animal pathology was detected in both negative and vehicle controls. Regarding AhR agonist compounds, no gross pathology was detected with PCB126, 3MC, AMB or β-naphthoflavone. While both AZ1c and TCDD could not kill any *C. elegans*, both chemicals managed to limit the movement of L4 animals at the highest concentration tested (1 nM for AZ1c and 1 µM for TCDD). It was clear that the movement of L4 *C. elegans* was sluggish in contrast to the negative control animals. On repeating the lethality test of both TCDD and AZ1c on AhR null *C. elegans* (CZ 24 85), the effects of both TCDD and AZ were abolished; the animals were moving freely and there was no difference from the negative control animals. This indicates that the *C. elegans* response to TCDD and AZ1c was mediated by CeAhR.

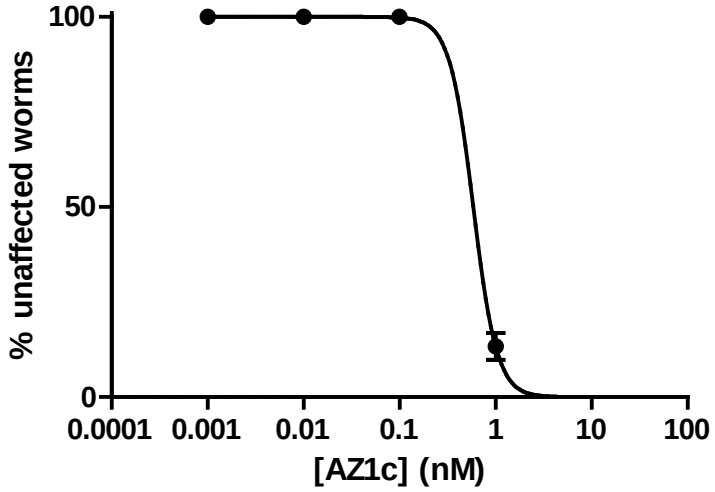


Figure 41 The effect of increased concentration of AZ1c in nM on the movement of wild type C. *elegans*. The movement of the animals was affected by only the highest soluble concentration of compound. The data points are means from three repeats. The curve fit is a four-parameter logistic equation giving an estimated IC_{50} of 0.59 nM.

3.12 Induction of CYP-GFP fused transgenic *C. elegans*

Strains of transgenic (CYP-GFP fused) animals (CYP35A2, CYP29A2 and CYP34A9) were subjected to induction by different known AhR agonists. This experiment aims to see if CeAhR can induce any CYP family genes as a model for humans. These 3 CYP enzymes are known to deal with xenobiotic responses in *C. elegans*. It is thought that one of them could correspond to human CYP1A1 and it could be induced by AhR agonists in *C. elegans*. The fluorescence (total) of these animals was measured by the dark reader machine aiming to detect the corresponding CYP enzyme induced by AhR (see Table 10). None of the fluorescence readings in the presence of the test compounds increased above that

of the control samples in their absence. This indicates that none of the CYP enzymes were induced by any of the compounds.

Table 10. Fluorescence of CYP-GFP fused transgenic *C. elegans* induced by serial dilutions of TCDD, AZ1c, 3MC, PCB126 and AMB). The *C. elegans* were exposed to the compounds for 3 days before measuring the amount of fluorescence.

Compound	Concentration	Fluorescence due to CYP induction		
		CYP35A2	CYP29A2	CYP34A9
TCDD	control	259	270	259
	0.001 µM	230	214	228
	0.01 µM	240	276	266
	0.1 µM	224	243	235
	1 µM	219	243	271
AZ1c	control	288	277	294
	0.001 nM	262	254	270
	0.01 nM	269	271	255
	0.1 nM	266	249	261
	1 nM	270	281	288
3MC	control	277	277	291
	0.02 µM	266	259	278
	0.2 µM	271	269	277
	2 µM	265	281	279
	20 µM	279	277	284
PCB126	control	288	299	291
	0.01 µM	265	277	270
	0.1 µM	268	270	268
	1 µM	271	271	269
	10 µM	259	288	288
AMB	control	269	259	278
	0.1 nM	270	270	266
	1 nM	268	281	276
	10 nM	259	271	269
	100 nM	294	277	280

4 DISCUSSION

AhR is a cytosolic receptor that binds multiple ligands (Zhou et al., 2010) and has a xenobiotic function in the metabolism of various toxic substances (Wang et al., 2009). AhR is also a target of many drugs like omeprazole and tamoxifen (Yoshinari et al., 2008; DuSell et al., 2010). AhR mediates the toxic effects of many pollutants including one of its most notable agonists, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is considered the full agonist of human AhR (Howard et al., 2010). Its stimulation induces non-genotoxic carcinogenesis (Cole et al., 2003; Schwarz and Appel 2005; Ellinger-Ziegelbauer et al., 2009). AhR stimulation creates a model for cancer in humans, and so understanding how AhR works becomes very important. Crystal structure studies on mouse and human AhR, and especially its ligand binding domain, resulted in poor outcomes (Lo Piparo et al., 2006). The 3-dimensional structure of AhR is important to understand how this receptor binds different structures and chemicals with different affinities, either agonists or antagonists. Despite the diversity between these ligands, it is not known how AhR binds to all these chemicals. Furthermore, AhR is considered as an orphan receptor, without a known endogenous ligand (Kung et al., 2009). The 3-dimensional structure of CeAhR is proposed to be a good template for creating a computer-based human AhR model.

This theory is attributed to the structural similarity between the ligand-binding domains of both proteins. The CeAhR LBD has the closest structure to the mammalian AhR compared to other non-mammalian species; the yielding protein domain shares nearly 50% similarity with human AhR LBD. It is suggested that expression, purification and crystallization of CeAhR would be easier than mammalian AhR, as it is thought to require less complex chaperones to fold, and it is expected to be an alternative to mammalian AhR crystal structure study. CeAhR is not known to bind ligands, but then its LBD is supposed to be less complex in structure, requiring less difficulty to be expressed in a soluble folded form. As a result, it is possible to obtain a human AhR model that is more compatible with the real 3-dimensional structure of AhR.

Furthermore, there is another debate about the function of CeAhR, whether it is a receptor or a transcription factor without a ligand pocket at all. Powell-Coffman and her group characterised AhR-1 in 1998, and tested its functionality by co-immunoprecipitation with rabbit HSP90, and confirmed that AhR-1 does not bind TCDD in vitro. The theory that AhR-1 LBD does not have a ligand-binding pocket makes its expression easier than human AhR. It is supposed that the AhR pocket collapsed during recombinant expression of mammalian receptors making crystallization difficult. Comparison between CeAhR and human AhR LBD revealed nearly 50% similarity; therefore, it is logical to use AhR-1 LBD domain as a template for human AhR with more accuracy than previous models proposed (Pandini et al., 2007; Pandini et al., 2009; Jogalekar et al., 2010). They used bHLH

templates with less than 30% similarity. The idea that CeAhR had no ligand-binding pocket and better similarity coupled to the probability of better expression generated hope of achieving a better human AhR model by attempting expression of CeAhR LBD.

The study showed that attempts to express CeAhR LBD in 3 different expression systems did not produce any significant quantities of purified soluble protein and far less than would be required for crystallization studies. The worst result was in the *Pichia pastoris* expression system, which expressed no protein at all. The results of expression of CeAhR LBD in the body muscle wall of transgenic *C. elegans* were very poor. The best expression system determined by this study was also the simplest, namely *E. coli*. The study expressed CeAhR LBD in both PL21(DE3) cells and Arctic express DE3 cells. The outcome of soluble CeAhR LBD was better with lower temperature expression in Arctic express cells. The problem was that the amount of soluble CeAhR LBD was too little. To get 1 mg of soluble CeAhR LBD, provided there was no loss during purification, would require 10 to 20 litres of bacterial culture. The study moved to answer the question, why CeAhR LBD expression was more difficult than expected. Experimental work was performed in *Pichia pastoris* but although it was possible to detect the RNA of the recombinant CeAhR LBD gene indicating successful transcription of CeAhR LBD inside the Pichia cells, no protein was produced.

Studies of the effect of known AhR ligands on both wild type *C. elegans* and AhR null live animals showed that CeAhR can no longer be considered a non-

xenobiotic binding domain. CeAhR responded to TCDD and AZ1c in the in vivo study. The study further tried to detect if CeAhR induced any of GFP.CYP35A2, GFP.CYP29A2 or GFP.CYP34A9 genes. These CYP genes are known to be induced in response to xenobiotic effects (Menzel et al., 2001). However, none of these genes were induced by TCDD or the other chemicals applied in this study.

AhR and ARNT genes in *C. elegans* were discovered by Powell-Coffman and her group in 1998. They discovered a DNA sequence coding for a protein that has structural similarity to the PAS domain of mammalian AhR. The group amplified this DNA segment that is obtained from a mixed-stage cDNA library, and named the gene AhR-1 (aryl hydrocarbon receptor-related). Fortunately, AhR-1 protein (CeAhR herein) shares 38% identity with human AhR over a region of 395 amino acids; furthermore, alignment showed that CeAhR is closely related to mammalian AhR in the PAS domains. What makes CeAhR more interesting is that it has similar biochemical properties to mammalian AhR (Bell and Poland 2000). CeAhR contains both bHLH and PAS domains like vertebrate AhR. Studies showed that this protein contains a PAS-B domain, which is the ligand-binding domain in mammalian AhR. On the other hand, the PAS-A domain of CeAhR shows ~56% amino acid identity with the corresponding sequence of mammalian AhRs. The bHLH sequence is less similar to the bHLH sequence in vertebrate AhRs. These observations suggest that CeAhR protein is a good model for mammalian AhR, as it has similar structural and biochemical properties (Hahn et al., 1997).

SDS-PAGE analysis of protein from His-tagged CeAhR LBD transformed BL21DE21(DE3) cells showed the expressed protein band at about 29 kDa, which would be expected for his-tagged CeAhR LBD and was the only difference from the antisense control. This experiment strongly implies expression of CeAhR LBD. Studies have been performed to express mammalian AhR in a baculovirus system and their outcome was also successful induction of AhR, yet the major obstacle was the fact that most of the expressed protein was insoluble (Chan et al., 1994).

The *E. coli* cells were sonicated to separate the soluble protein content, and also centrifuged at 10,000×g to remove the insoluble protein. The soluble component was further centrifuged at 250,000×g. The protein bands were compared to the pelleted Prset transformed BL21(DE3) cells. Protein sequencing of soluble fractions confirmed that the CeAhR LBD protein was induced and present. CeAhR LBD was also transformed into Arctic Express Cells. Both BL21(DE3) and Arctic Express DE3 cells use the strong T7 promoter. However, Arctic Express cells expressed more soluble CeAhR LBD at lower temperature (12°C). The problem of recombinant expression in *E. coli* is too fast production of a large amount of recombinant protein that accumulates in the inactive form of inclusion bodies. These require a lot of effort to refold, and even if successful, it is difficult to guarantee that the refolded CeAhR LBD protein is biologically active to study its structure. Arctic Express cells grow at low temperature giving good opportunity to express soluble AhR. Furthermore, Arctic Express cells have been modified to

express chaperones cpn10 and cpn60. These chaperones could stabilize the recombinant protein tertiary structure and express more functional protein. Indeed, Arctic Express results were better than PL21(DE3), but the overall soluble protein expression was still poor.

4.1 Solubility of the protein

SDS-PAGE analysis of soluble His-tagged CeAhR LBD, after purification within a nickel column, was performed. The results of the column protein bands were compared with serial dilution of BSA to evaluate the amount of purified protein. Unfortunately, the amount of the protein was too little, and seen only in the wash samples of the column. No protein was detected in the eluted samples. Even though soluble protein could be obtained from the bacterial expression system without complex chaperone protein, the amount of soluble protein is small. It would take more than a 10 litre culture of *E. coli* to purify less than 1mg of soluble His-tagged CeAhR LBD. For large-scale culture, purified CeAhR LBD expression, we would need tens of litres or more of *E. coli* culture. Studies showed that high throughput (HTP) expression of *C. elegans* proteins in *E. coli* processed 10,167 different *C. elegans* genes. 4854 (47.7%) proteins were successfully expressed, and of these, 1536 (15.1%) were soluble proteins. Only 590 proteins were expressed at a large enough scale for crystal structure studies (Finley, 2004). Cytoplasmic proteins are less easily secreted than membrane proteins, and homomeric proteins are less soluble too. The presence of a large number of

cysteine amino acids is a bad indicator of protein solubility (Luan et al., 2004). CeAhR LBD has few cystiene residues which is a good sign, however most of the expressed CeAhR LBD in bacteria was insoluble; it is noteworthy that AhR is a cytosolic protein.

4.2 GST and poly histidine tag fusion proteins

The study indicated that CeAhR LBD should be fused with GST and poly histidine tags. The major aim of both tags was to make the purification of CeAhR LBD simpler. It is worthy to compare both tags regarding expression. Also, the comparison between the 2 different proteins' crystal structure will support the credibility of AhR structure analysis; in other words, consistent AhR crystal structure, despite different fusion tags, indicates robust AhR folding. On the other hand, the fusion protein tags interfere with the biochemical study of the expressed protein. The presence of the fusion tag protein at the start of translation of the protein may express less recombinant protein as the first amino acid sequence is important in protein signalling. The presence of the tag protein at the end of the target protein may be a better option to improve the expression of the protein.

4.3 AhR Expression in Pichia pastoris

The yeast expression system has many advantages that make it a good choice for CeAhR LBD expression on a large scale. *Pichia pastoris* is a eukaryotic system that can carry out post-translational modifications by virtue of its endoplasmic

reticulum. So the soluble, expressed protein secreted by that system is supposed to be functional. This is a very important advantage, because CeAhR is not known to have a specific ligand with which to assess its functional capabilities. A major question arises here: how would CeAhR LBD be assessed in a functional way? First, it would be soluble, second, it would not be aggregated, and third, if it could be crystallized and its structure solved, it will be fairly obvious if it is a PAS protein fold or not. Finally, successful CeAhR LBD secretion by the yeast expression system will indicate functional post-translational modification. The *Pichia pastoris* system is supposed to have more complex chaperones that could help in expressing more soluble CeAhR LBD. Other advantages offered by the yeast expression system include: 1. Yeast yields a high level recombinant protein due to the presence of a large copy number of vectors per cell. 2. The promoter is strong and stable. 3. The system is characterised by high cell density. 4. The yeast system can produce dry-cell weight densities that exceed 100 gram/litre. 5. Yeast requires growing media which is simple and inexpensive. 6. As yeast is eukaryotic, it is capable of expressing soluble, complex mammalian proteins. 7. Recombinant plasmids are integrated in the chromosomal DNA of the yeast genome making the recombinant DNA stable for different generations. 8. Yeast expression is durable, and requires no complex care. It is, therefore, usually very suitable for generating a large amount of functional protein for 3-dimensional structure studies.

In this study expression was mediated by the AOX1 promoter (Cereghino and Cregg 2000; Cregg et al., 2000; Lin Cereghino et al., 2001). Dot blot screening of

100 colonies of His-tagged CeAhR LBD transformed Pichia were studied, but only one colony gave positive results. The whole 100 colonies of GST CeAhR LBD gave no signal at all. It seems that achieving CeAhR LBD expression in *Pichia pastoris* is a difficult task. It is important to say that dot blot screening may yield false positive results and it is essential to further analyse the Pichia colonies by western blot. The dot blotting screened the possible secreted CeAhR LBD in the surrounding media. The cornerstone of success in that expression system is the ability to secrete CeAhR LBD protein even in minute amounts. Secreted protein should be subjected to post-translational modification, and supposed to be a functional and good candidate for crystal structure work. The western blot results concluded that the expression of CeAhR LBD was very poor, both secreted and accumulated intracellularly. The unfortunate results of the secreted pPICZ alpha B may be explained by the theory that CeAhR LBD is a cytosolic protein and tends to stay intracellular. It was hoped that Pichia could secrete even nanogrammes of CeAhR LBD protein, and later on the biomass is concentrated making a number of litres yield a few mg of protein, yet no CeAhR LBD was secreted at all. The positive results of the dot blot in one colony were clearly a false positive. Furthermore, testing for intracellular CeAhR LBD protein revealed similar poor results. The western blot failed to detect any CeAhR LBD expressed by *Pichia pastoris*. Despite the expectation that the yeast system might be more productive than the bacterial system, clearly the opposite was true. It is quite difficult to predict the results of expression systems in advance.

The first step to track protein manufacturing is checking for successful DNA integration into the genome of the yeast. The genomic DNA of the recombinant yeast was extracted and amplified by PCR reaction targeting the recombinant DNA. It showed that the DNA of CeAhR LBD was successfully integrated.

The total RNA was extracted from *Pichia pastoris* transformed by CeAhR LBD constructs. The RNA was reverse transcribed to cDNA which was amplified by PCR reaction and the PCR product showed the CeAhR LBD gene in agarose gel. This showed that there was no problem with transcription of CeAhR LBD inside *Pichia pastoris*. Therefore, the problem may be in the translation process or after translation. After translation the recombinant protein is subjected to post-translation modification in the eukryotic system which is supposed to result in folded protein.

The process of recombinant protein expression could be intrupted at any of these stages. On the other hand all the above process may be successful and the protein may be subjected to degradation. It is important to remember that the pH and redox state may affect the production of recombinant proteins in different expression systems favouring one protein over another. In this case the problem occurred downstream of mRNA transcription but to precisely determine which further step failed would be a major undertaking.

4.4 AhR Expression in transgenic C. elegans

The third trial was to try and express CeAhR LBD in transgenic *C. elegans* itself. The CeAhR LBD is supposed to be expressed with both GST and His tags in the muscle wall of *C. elegans*. The protein expression was mediated by the UNC 54 promoter. The CeAhR LBD DNA was microinjected with M cherry marker. The problem was that M cherry uses the same promoter as the pPD30.38 constructs of CeAhR LBD. Other markers not using UNC 54 have been tried, but were toxic to C. elegans. The CeAhR LBD expression results were poor also. *C. elegans* failed to express CeAhR LBD in the muscle wall, although transgenic *C. elegans* should contain all the chaperones needed to manufacture its own protein or at least the full length CeAhR. It is important to say that the target protein is only a domain of a larger protein and may lack the regions necessary for interactions with chaperones. It may also be that this protein is considered foreign as it is expressed with foreign tags in the muscle wall, which is not the normal intracellular environment. However, PAS domain proteins have been successfully expressed independent to the N-terminus region. It is not known whether the expression of the full length CeAhR would be more successful than just the LBD.

4.5 Comparison of expression systems

So, three trials of CeAhR LBD expression were performed with disappointing results. The best expression system was Arctic Express cells. It is difficult to know why some protein expression succeeds in one system and fails in another. For

example, promoter strength, pH, local ionic environments, redox state, copy numbers, transcript levels, codon bias, chaperone levels or chaperone affinities may be important factors that decide the success of one system or another for a particular protein (Herrero and Sentandreu 1988; Gregory A Petsko 2009). The expression of CeAhR LBD was best at lower temperature (12 ^{o}C).

4.6 *C. elegans* AhR endogenous function

The endogenous function of CeAhR was studied by Qin and Powell-Coffman in 2004. It is known that CeAhR is strongly related to the neurological maturation of *C. elegans*. Binding assays showed that TCDD was not binding to CeAhR expressed in vitro. It is not known if CeAhR functions as a receptor in vivo, or works as a transcription factor without any ligand at all. In this study an experiment was carried out to determine whether any known AhR ligands affect live *C. elegans*. Lethality tests were performed with 6 different known mouse AhR ligands from different groups on N2 *C. elegans*. The results showed that all 6 compounds have no lethality effects on N2 wild type *C. elegans*. However, both TCDD and AZ1c had a behavioural effect on wild type animals. At the maximum soluble level, both compounds induced limitations in the movement of the animal in contrast with negative control *C. elegans*. The experiment was repeated 3 times with the same finding. These limited effects of ligands are attributed to the weak solubility of TCDD and AZ1c. However, it implies that *C. elegans* contains active AhR that binds TCDD in mice and humans. A very important question arises here.

Is the CeAhR LBD responsible for these new findings or is another gene with different receptor protein binding TCDD? It is known that Zebra fish contains 2 AhR proteins, namely AhR1 and AhR2, and only one of them actively binds TCDD. In order to solve this problem, the in vivo tests were repeated with TCDD and AZ1c with CeAhR null animals, i.e. (CZ 85 24) strains. These strains have no functioning AhR-1. The results revealed completely functioning animals that are identical to the control, that is to say that AhR null *C. elegans* abolished the effect of TCDD and AZ1c. This indicates that the CeAhR LBD could bind ligands that are active at mammalian AhRs.

The study in 1998 by Powel-Coffman indicated that CeAhR does not bind ligands. However, this in vivo study indicates the opposite. In Powell-Coffman's (1998) study, they checked protein functionality by active binding with HSP90. It seems that HSP90 binds to a pocket away from the AhR LBD, and this contrasts with the previous data in the literature (Fukunaga et al., 1995). It assumed that CeAhR was fully active because, like mouse or human AhR, it bound HSP90. Recombinant CeAhR binds HSP90, but not TCDD (Powell-Coffman et al., 1998). However, this in vivo study says that CeAhR binds TCDD. This may be explained by saying that ligands and accessory proteins bind to two different sites on AhR. So presumably the protein was not functional in terms of ligand binding when it was used in the in vitro study but it is in vivo. From this work it can be said that protein folding in vivo is different from that in vitro. This result can be applied for different proteins.

Previous studies claimed that the PAS B domain is the place where HSP90 and the proposed ligand combine with the LBD (Pandini et al., 2007). It is suggested that this model is not accurate for C. elegans. It seems that AhR has multiple binding sites where chaperones and ligands bind. These results support some theories claiming that multiple ligands bind to multiple sites (Henry and Gasiewicz 2008). These models show only one cavity for ligand binding and depend on templates with similarity of less than 50%. Therefore, there are some doubts about the accuracy of these models (Lo Piparo et al., 2006; Pandini et al., 2007; Bisson et al., 2009; Jogalekar et al., 2010). A lack of success in crystal structure studies of any AhR protein makes such models ambiguous. Also, there is no data on the quaternary structure of AhR, whether AhR is a monomer, dimer, or multi-subunit protein. From the evolutionary point of view, it was thought that invertebrate AhR was a transitional stage before full maturation of the function in humans. However, this statement may not be true. CeAhR is advanced in function and binds xenobiotic ligands like vertebrates, and thus its structure is supposed to be as complex as human or mouse AhR. This may be why the expression of CeAhR was difficult, as it is in humans and mice.

4.7 Induction of cytochrome P450 analogues in *C. elegans*

Three strains of C. elegans were used to test for induction of several CYP genes. The strains were GFP-CYP29A2, GFP-CYP35A2 and GFP-CYP34A9. The results concluded that ligand bound CeAhR does not induce these 3 cytochrome P450

enzymes. However, there are more than 60 such enzymes in *C. elegans*. Microarray analysis on AhR-mutated L1 stage *C. elegans* showed that 324 genes were down-regulated and 238 genes were over-expressed; these genes were related to fatty acid metabolism, growth and development (Aarnio et al., 2010). Previous studies showed that β-naphthoflavone has been found to induce CYP35A2 (Menzel et al., 2001; Schafer et al., 2009). However, in the present study, 5 different AhR agonists (TCDD, AZ1c, 3MC, PCB126, AMB) did not induce any of CYP35A2, CYP29A2, or CYP34A9. This may be explained by multiple pathways of metabolism for different chemicals in *C. elegans*. The AhR agonist chemicals used in this study are poorly soluble in water, and it may be that the concentration of these chemicals was not strong enough to induce CYP35A2 grossly. On the other hand, the lethality test experiment of β-naphthoflavone on wild type *C. elegans* showed resistance to the toxicity of this compound at the highest soluble concentration. It is not known if β-naphthoflavone is an AhR ligand in *C. elegans*, and so this drug may be metabolised in a different manner or it induces CYP35A2 at a low level that could not be detected by the assay used here. The study performed by Menzel et al. (2001) induced CYP in transgenic *C. elegans* that contain transgenic CYP35A2 in the intestine. They detected the induction by visualising the muscle wall florescence, but not measuring the total absorbance from the animal. It is not known if CeAhR induces any of the CYP family in *C. elegans* that are homologous to those in mammals.

4.8 Future work

If it is possible to grow 50 litres of E. coli (Arctic Express DE3) this could yield about 5 mg of soluble CeAhR LBD that could be subjected to crystal structure study, or at least protein characterization with different protocols, for example analytical ultra centrfugation. 1 mg of the soluble CeAhR LBD can provide a lot of data about the configuration of CeAhR that could help further studies. It is important to say that CeAhR LBD is a part of a protein. Expression of the whole CeAhR protein is another project. It is not known if the expression of the whole protein may result in better folded AhR with better solubility. It is possible to conjugate CeAhR with both GST and GFP tags at the same time in a new study to enhance the protein's solubility and at the same time help purification easily; the GFP would help protein solubility (Gonzalez-Montalban et al., 2007) and GST tag would help in the purification of CeAhR. It is also possible to study more CYP family members fused with GFP to see if any of these are induced by CeAhR activation. This is not easy work, because *C. elegans* has 60 different CYP family enzymes. In this case, it would be sensible to apply computer-based homology determinations to find the most homologous CYP enzyme in *C. elegans* to CYP1A1 in mammals. It is possible later on to fuse this CYP enzyme with GFP and perform an induction study on it. It is important to remember that it is not clear if *C. elegans* has a xenobiotic metabolic function as in humans, although the data herein implies that it does. It is possible in the future to try more compounds

and more types of behavioural assay, for example, brood size assay, growth assay and feeding assay.

4.9 Conclusion

It can be concluded from this study that CeAhR has similar criteria to the mammalian AhR. It binds xenobiotic ligands as it does in mammals but perhaps with lower affinity. Therefore, crystal structure work of that protein is more relevant than previously thought. On the other hand, difficulty in expressing CeAhR would be expected as in other species. The success in expressing a very small amount of soluble CeAhR LBD in bacteria is encouraging to continue this work in future with larger scale facilities. Moreover, there is hope to achieve the target of this project in performing a crystal structure study of CeAhR either LBD or the full length protein. It should be emphasised that CeAhR is a receptor that binds ligands, and it is important to study the battery of genes affected by CeAhR. *C. elegans* could be a good model for dioxin toxicity and cancer evolvement that could help in understanding AhR biology. It is worthy to say that *C. elegans* is still a good model for human AhR 3-dimensional structure, despite the difficulties in application.

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