CURCUMIN REGULATES TRANSCRIPTION IN *DICTYOSTELIUM DISCOIDEUM*

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

CURCUMIN REGULATES TRANSCRIPTION IN *DICTYOSTELIUM DISCOIDEUM*

presented by William S. Swatson III,

a candidate for the degree of Doctor of Philosophy

and hereby certify that, in their opinion, it is worthy of acceptance.

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This dissertation is dedicated to my father and the loving memory of my mother for

their love, support, encouragement and sacrifice.

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A. *Dictyostelium discoideum*

I. *D. discoideum* as a model organism

The haploid cellular slime mold, *Dictyostelium discoideum*, is a eukaryotic soil-dwelling amoeba. It has been used as a model organism to study complex biological processes due to its short and simple life cycle with discrete unicellular and multicellular stages. *D. discoideum* has the ability to progress through a well-defined multicellular developmental cycle, during which it differentiates into two distinct cell types - spores and stalk cells (Loomis, 1975). The entire genome of *D. discoideum* and a variety of related species have been fully sequenced (Eichinger et al., 2005; Heidel et al., 2011; Sucgang et al., 2011). *D. discoideum* has proven to be very useful in studies addressing cell motility, cell differentiation, chemotaxis, cell type specific gene expression and development (Loomis, 1975). In addition, it now has been used extensively for pharmacogenetic studies (Alexander et al., 2006; Williams, 2005). Overall, there is over 80 years of accumulated research (approximately 10,000 publications) on this remarkable organism, and the research community is supported by an NIH funded stock center and genome database.

II. Life cycle of *D. discoideum*

The most distinguishable feature of *D. discoideum* is its life cycle, alternating between vegetative growth by mitotic cell division and multicellular development (Fig 1.1). *D. discoideum* grows mitotically, feeding on bacteria or defined medium. The cells remain individual as long as nutrients are available to them. However, when the food source is depleted, the developmental cycle is initiated. Individual cells release the chemoattractant, cyclic adenosine monophosphate (cAMP) into their environment and neighboring cells respond by chemotaxing toward the signal, and relaying the signal outwards ultimately forming multicellular aggregates of 10^5 cells (Devreotes et al., 1979). Cells within the multicellular tissues then differentiate into either prespore (80%) or prestalk (20%) cells. The prespore cells in each multicellular aggregate ultimately differentiate into spore cells while the remaining 20% prestalk cells differentiate into stalk cells. The combination of chemotaxis, cell-to-cell adhesion, cellular signaling, cell differentiation and multicellular morphogenesis makes *D. discoideum* an excellent model for development.

III. Molecular genetics of *D. discoideum*

D. discoideum has about 12,000 protein coding genes, many of which share sequence similarity with higher eukaryotes. The genes reside on six haploid chromosomes with a total genome size of 34 Mb and are separated by intergenic regions of 2.5 kb on average. Intergenic regions are very A/T rich (85%). Most genes have one intron although some have multiple introns or none at all. Introns are usually very small
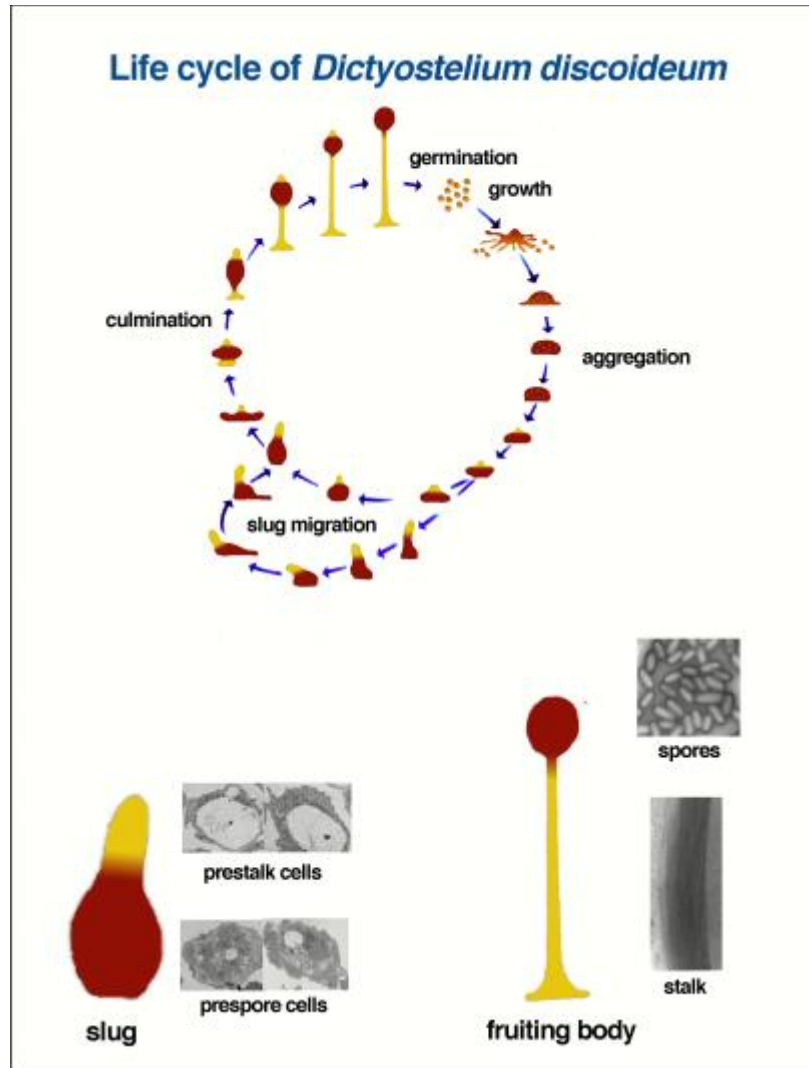
Fig 1.1: Life cycle of *Dictyostelium discoideum*. Mitotically dividing cells aggregate to form multicellular aggregates upon depletion of nutrients. Over 24 hours, aggregates of 100,000 cells, undergo a well defined and synchronous sequence of gene expression, cell differentiation and morphogenesis to form a fruiting body (80,000 spores) on top of a stalk comprising 20,000 stalkcells. Aggregates may form a migrating slug, moving along light and heat gradients eventually resuming development depending on favorable conditions.

with an average of 200 nucleotides. Besides the vegetative mitotic division, *D. discoideum* also has a sexual cycle, although it is not experimentally tractable (Kessin, 2010). Before gene cloning techniques became available, parasexual genetics were used to map mutations at low resolution (Sussman and Sussman, 1963). Haploid cells, each with a different growth temperature-sensitive mutation were fused to form temperature-resistant diploids. Haploid segregants from spontaneous haploidization could be isolated if recessive drug resistant markers were included in the diploids, and the markers/phenotypes were mapped to specific chromosomes (Loomis, 1982). There are sets of molecular tools and techniques available today that make *D. discoideum* an excellent model for biochemical and physiological studies. Specific genes can be disrupted by homologous recombination (Faix et al., 2004). Insertional mutagenesis allows for the unbiased identification of specific genes responsible for a particular phenotype and the creation of isogeneic cell lines (Kuspa and Loomis, 1992).

IV. *D. discoideum* as a model for human disease

Early analyses of the *D. discoideum* genome sequence under very stringent conditions identified 64 human disease genes (Eichinger et al., 2005) and others have since been added (Alexander et al., 2006). In addition, *D. discoideum* shares many signal transduction pathways with mammals. The Rho family of GTPases is a family of signaling G proteins that regulates cell polarity, cell morphology and the cell cycle (Bustelo et al., 2007). In *D. discoideum*, Rho GTPases have been shown to regulate cell morphology, endocytosis, cytokinesis, cell polarity and chemotaxis (Rivero and

Somesh, 2002). Another example of a signaling pathway that *D. discoideum* shares with mammals is the cAMP-protein kinase A (PKA) dependent pathway important for early development (Firtel and Chapman, 1990).

One major area of research demonstrated PIP_3 signaling as a cellular target in *D. discoideum* for the mood-altering drug, lithium (King et al., 2009). Suppression of PIP_3 signaling by lithium in *D. discoideum* was subsequently validated in humans. This same group has continued to use *D. discoideum* to search for new targets and pathways for taste reception (Robery et al., 2013). This study identified the metabotropic glutamate receptor-like gene, *grlJ,* as being responsible for resistance to the commonly used bitter standard, phenylthiourea.

Other studies have also successfully used *D. discoideum* to reveal new targets for anti-cancer drugs.   Specifically, insertional mutagenesis studies produced mutants that were more resistant to the widely used anti-cancer drug cisplatin (Li et al., 2000). This and subsequent studies (Min et al., 2004; Min et al., 2005a) demonstrated that sphingosine-1-phosphate (S-1-P), by virtue of its intracellular signaling properties, is a key regulator of sensitivity to cisplatin and a similar drug carboplatin, but not drugs that have different mechanisms of action. This genetic and biochemical framework that was revealed by the studies on *D. discoideum* was then translated and validated in studies on human cells (Min et al., 2007; Min et al., 2005b). The operative mechanisms by which S-1-P regulated cisplatin sensitivity were shown to be virtually identical in several human cells line, and these studies were extended to six human

ceramide synthases, which synthesize ceramide, another bioactive sphingolipid that was thought to be antagonistic to S-1-P. Indeed, it was shown that the relative levels of ceramide and S-1-P regulate the sensitivity of human cells to cisplatin. It was further demonstrated that sensitivity to platinum drugs could be altered pharmacologically with inhibitors of sphingosine kinase and it was suggested that this could be used clinically to improve the efficacy of cisplatin (Alexander and Alexander, 2011; Alexander et al., 2006).

Overall, these pioneering studies have clearly demonstrated the utility of *D. discoideum* for understanding the mechanism of action of drugs in humans. The identification of single genes by insertional mutagenesis leads to the identification of entire signaling pathways, and thus multiple molecular targets for potential therapeutic intervention.

By taking advantage of this simple but powerful biological model and the molecular genetic tools available to us, we have started to investigate the complex effects of the botanical compound curcumin on cell physiology.

B. Botanical drugs and natural remedies

I. Botanicals and their use in therapeutics

The use of botanicals is becoming increasingly popular. According to the World Health Organization (WHO), it is estimated that 80% of the world's population use botanicals as part of their primary health care. In the United States, 20% of Americans

use botanicals, with billions of dollars spent each year on these natural remedies (Bent, 2008). In fact, according to a report published by Persistence Market Research, the global botanical market was valued at $54.6 billion dollars in 2013 with a forecasted market value estimated to reach $90.2 billion by 2020 (Research, 2014).

One notable herbal remedy that has contributed significantly to the advancement of healthcare is the antimalarial drug, artemisinin. According to the WHO, malaria, caused by the *Plasmodium* parasite, is present in about one hundred countries and was the cause of an estimated 584,000 deaths, mostly in children aged under 5, in 2013. Artemisinin is a compound derived from the herb, *Artemisia annua*. It was discovered by Tu Youyou, winner of the 2015 Nobel Prize in Physiology or Medicine, during a plant screening research program in 1967 by the Chinese army to find a cure for malaria. Today, the world's most effective and quickest treatment of *Plasmodium falciparum* are artemisinin combination therapies (White, 1997).

Garlic has also been studied and the active ingredients, allicin (diallyl-dithiosulfinate), diallyl disulphide, S-allylcysteine and diallyl trisulfide have been identified (Abdullah et al., 1988; Lanzotti et al., 2012; McRae, 2005). Although garlic is a herb best known for culinary purposes, it can be used for medicinal purposes, some of which are supported by scientific evidence - for example, the treatment of high blood pressure (Al-Qattan et al., 2003; Benavides et al., 2007; Mohamadi et al., 2000; Reinhart et al., 2008), high cholesterol (Chowdhury et al., 2002; Yeh and Liu, 2001) and heart disease (Borek, 2001).

For many other botanicals including curcumin, the efficacy and safety have not been well established, and little or nothing is known about the underlying molecular mechanisms of action. Clearly this is a field where more work and innovation are needed.

II. Curcumin

1. A Brief History

Curcumin, a polyphenol, is the active ingredient (2 – 8 %) in the dried ground rhizome of the perennial herb, *Curcuma longa*, otherwise known as turmeric (Huang and Beevers, 2011). It has widely been used in traditional medicine, especially in India, China and Thailand. It has traditionally been used to treat a variety of diseases including anorexia, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis (Jayaprakasha et al., 2006). Curcumin is also very popular in the 5000-year-old Indian system of natural medicine known as Ayurveda, the science of life (Ayur=life, Veda=science or knowledge; Sanskrit). This system of medicine is entirely holistic, with its precedents set in the ability to maintain a balance in body, mind and spirit. It is devised to offer guidelines on daily routines, diet, conduct and the proper use of one's senses. Among curcumin's uses in the Ayurveda system of herbal medicine is the relief of insect bites and scratches, digestion improvement, cleansing and strengthening the liver and gallbladder and to eliminate worms. Curcumin is also used as a food colorant and dye for cloth because of its bright yellow-orange pigment. Curcumin was first isolated in 1815 by H.A Vogel and J. Pelletier, however, it took another century to determine its chemical structure and synthesis (Gupta et al.,

2012). Research over the years has suggested curcumin to have a wide spectrum of apparent pharmacological effects including anti-carcinogenic, anti-inflammatory, Alzheimer's prevention and antioxidant activity thereby wielding medicinal benefits against diseases such as cardiovascular diseases, neurodegenerative diseases, inflammatory bowel diseases, multiple sclerosis, arthritis, asthma and more. According to the United States Patent and Trademark Office, about 150 curcumin related patents have been registered since 1976. Curcumin's therapeutic effects have been attributed to its chemical structure, physical, biological and chemical properties but detailed molecular mechanisms are still unknown.

2. Chemical Properties of Curcumin

Curcumin has a molecular weight of 368.37 and a melting point of 183°C. Curcumin [1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,3-dione] is a diferuloyl methane molecule containing two aromatic o-methoxy phenolic groups separated by a seven carbon linker consisting of an α, β-unsaturated β-diketone moiety (Priyadarsini, 2013) (Fig 1.2). Curcumin is insoluble in water but dissolves readily in dimethyl sulfoxide, acetone and ethanol, and can be delivered to cells in these solvents. Studies on the degradation of curcumin under various pH conditions have shown its stability to be pH dependent (Tonnesen and Karlsen, 1985; Wang et al., 1997b). Curcumin is unstable in neutral to basic conditions with about 90% degrading within 30 minutes to *trans*-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloyl methane and vanillin (Lin et al., 2000; Wang et al., 1997a) (Fig 1-3). Degradation in basic conditions, however, is blocked with the
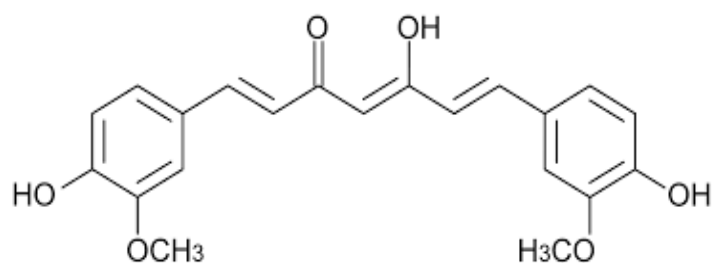
curcumin

Fig 1.2: Structure of curcumin. Curcumin [1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,3-dione] is a diferuloyl methane molecule. It is made up of two aromatic ring systems containing o-methoxy phenolic groups and connected by a seven carbon linker consisting of an α, β-unsaturated β-diketone moiety.

| *trans*-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal | Ferulic aldehyde | Ferulic acid | Feruloyl methane | Vanillin |
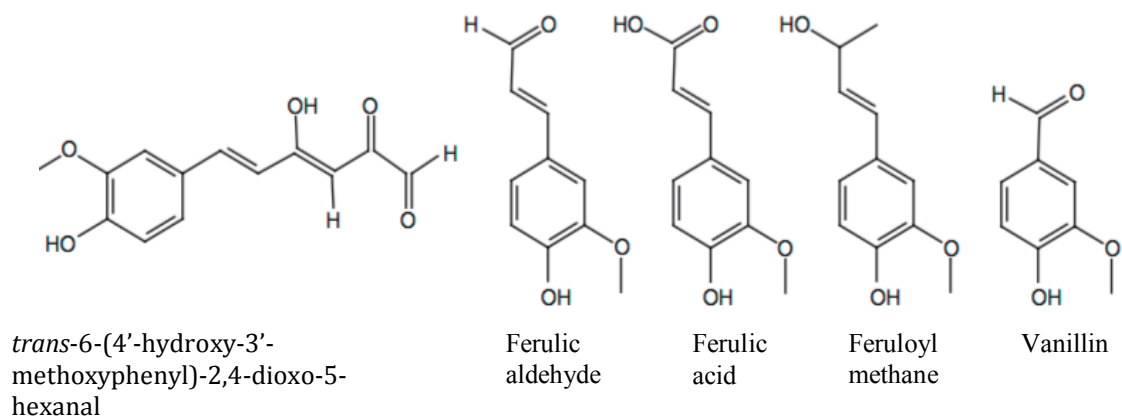
Fig 1.3: Degradation products of curcumin: Curcumin can degrade under various pH and physiological conditions. Degradation products of curcumin have been identified as *trans*-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic aldehyde, ferulic acid feruloyl methane and vanillin.

addition of antioxidants such as ascorbic acid, N-acetylcysteine, glutathione or in the presence of fetal calf serum or human blood (Wang et al., 1997b). Curcumin also degrades in acidic conditions, except at a much slower rate of 20% in 1 hr. (Wang et al., 1997a).

3. Molecular Targets of Curcumin

Previous studies have shown curcumin to be a highly pleiotropic molecule that has the ability to interact with a multitude of molecular targets (Fig 1.4). Curcumin is believed to affect the growth, development and spread of cancer by interfering with multiple cellular processes including cell cycle, apoptosis, proliferation, angiogenesis and metastasis (Anand et al., 2008; Conney et al., 1991).

Activated protein-1 (AP1) is a transcription factor which is a dimeric complex composed of several proteins belonging to the c-Fos, c-Jun, ATF and Jun dimerization protein families (Vesely et al., 2009). AP-1 functions in regulating cell proliferation, survival, apoptosis, differentiation and transformation (Karin et al., 1997). This function of AP-1 is in response to various stimuli including cytokines, growth factors, stress, bacterial and viral infections (Hess et al., 2004). AP-1 transcription factor binds to genes containing the 12-*O*-tetradecanoylphobyl-13-acetate (TPA), (TRE; 5'TGAG/CTCA-3') DNA response element sequence and enhances transcription (Angel et al., 1987; Hess et al., 2004). Curcumin inhibits AP-1 by directly binding to the DNA binding motif of AP-1 (Bierhaus et al., 1997).
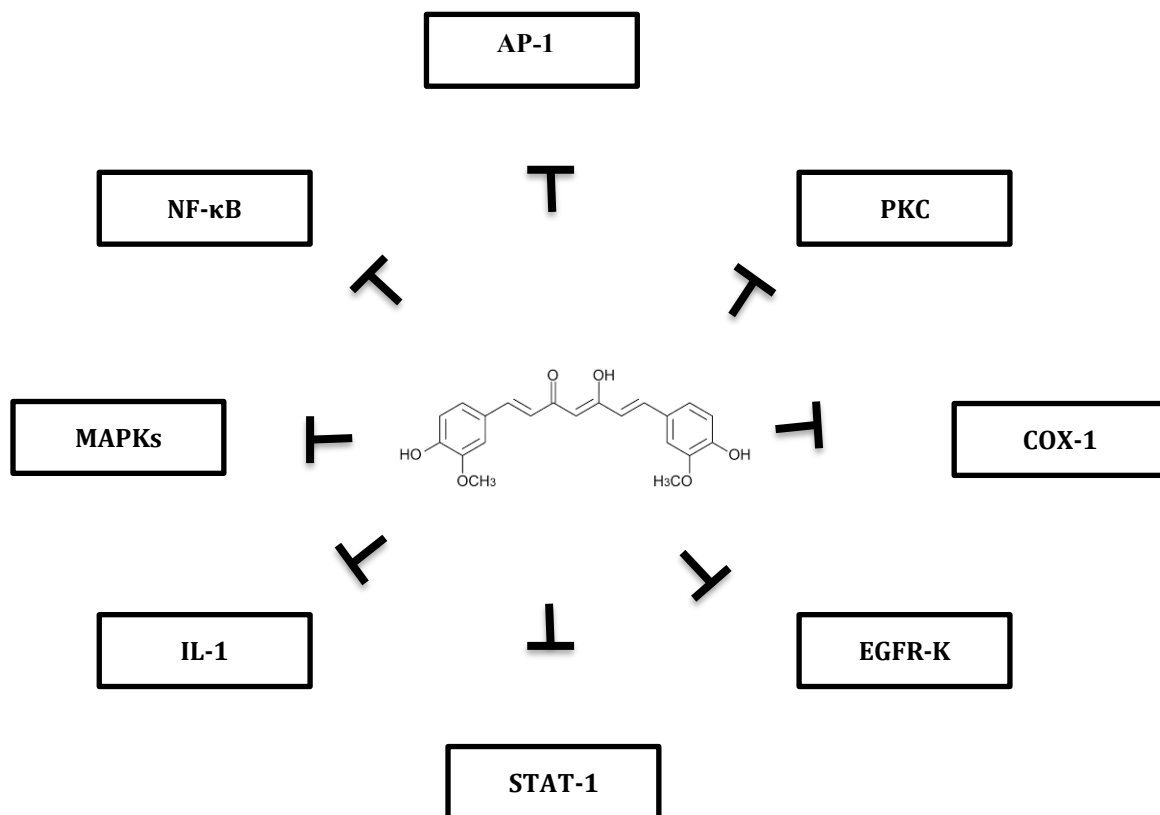
Fig 1.4: Molecular targets of curcumin. Studies on curcumin have suggested several molecular targets including enzymes, transcriptition factors and kinases.

Alzheimer's disease (AD), the most common neurodegenerative disease in the world, is characterized by a buildup of protein tangles in the brain known as amyloid plaques. Patients with AD have defects in the phagocytosis of amyloid beta (1-42) *in vitro* by macrophages and in clearance of amyloid beta plaques (Hardy and Selkoe, 2002; Zaghi et al., 2009). Macrophages in blood taken from AD patients treated with curcumin were shown to have improved uptake and ingestion of plaques over patients whose macrophages were not treated with the compound, indicating that curcumin has the ability to support the immune system in clearing amyloid protein (Zhang et al., 2006).

Curcumin has been shown to affect the inflammatory response and inflammatory diseases in humans. As revealed by *in vitro* studies, curcumin's inhibition of inflammatory cytokines is achieved in a number of ways. Curcumin has been shown to down regulate the activity of cyclooxygenase 2, lipoxygenase and inducible form of nitric oxide synthase enzymes through the suppression of NF-κB (Surh et al., 2001). Curcumin's suppression of the inflammatory response has also been attributed, in part, to the compound's suppression of the JAK-STAT signaling cascade through activation of Src homology 2 domain-containing protein tyrosine phosphatases (SHP)-2, a negative regulator of JAK activity (Kim et al., 2003). Curcumin also inhibits the production of TNF-σ and interleukin-1,-2,-6,-8 and -12 through inhibition of NF-κB and the MAPKs pathways (Cho et al., 2007). Inhibition of these cytokines was attributed to curcumin's ability to suppress MAPKs and NF-κB. In fact, studies have

shown curcumin to have high efficacy and fewer side effects compared to other anti-inflammatory drugs (Lal et al., 1999; Takada et al., 2004).

Perhaps the most frequent role attributed to curcumin is its antioxidant effect. Oxidative damage, a mechanism associated with the reaction of free radicals with organic substances such as lipids, proteins and DNA is thought to be the cause of many diseases and aging (Harman, 1956). Curcumin has been reported to act as an antioxidant to counter the effects of free radicals (Kunchandy and Rao, 1990). Antioxidant effects of curcumin have been shown in rat peritoneal macrophages (Joe and Lokesh, 1994) and in red blood cells (Tcnnesen and Greenhill, 1992).

Despite the growing interest in the use of botanicals including curcumin in disease treatment and prevention, and a growing literature of curcumin being used as a treatment for a myriad of diseases (Fig 1.5), there is little evidence regarding its long-term effects. There is limited research supporting efficacy and safety of curcumin, the fundamental molecular mechanisms associated with the cellular response to curcumin are not clearly understood, there are conflicting reports about its mechanism of action, and there is the likelihood of unknown off-target effects. For example, in addition to the evidence suggesting that curcumin is an antioxidant, recent reports suggest that curcumin possesses pro-oxidant effects, increasing reactive oxygen species (ROS) and thereby inducing oxidative stress within cells (Khan et al., 2012; Yoshino et al., 2004). Thus, there is a strong rationale for further
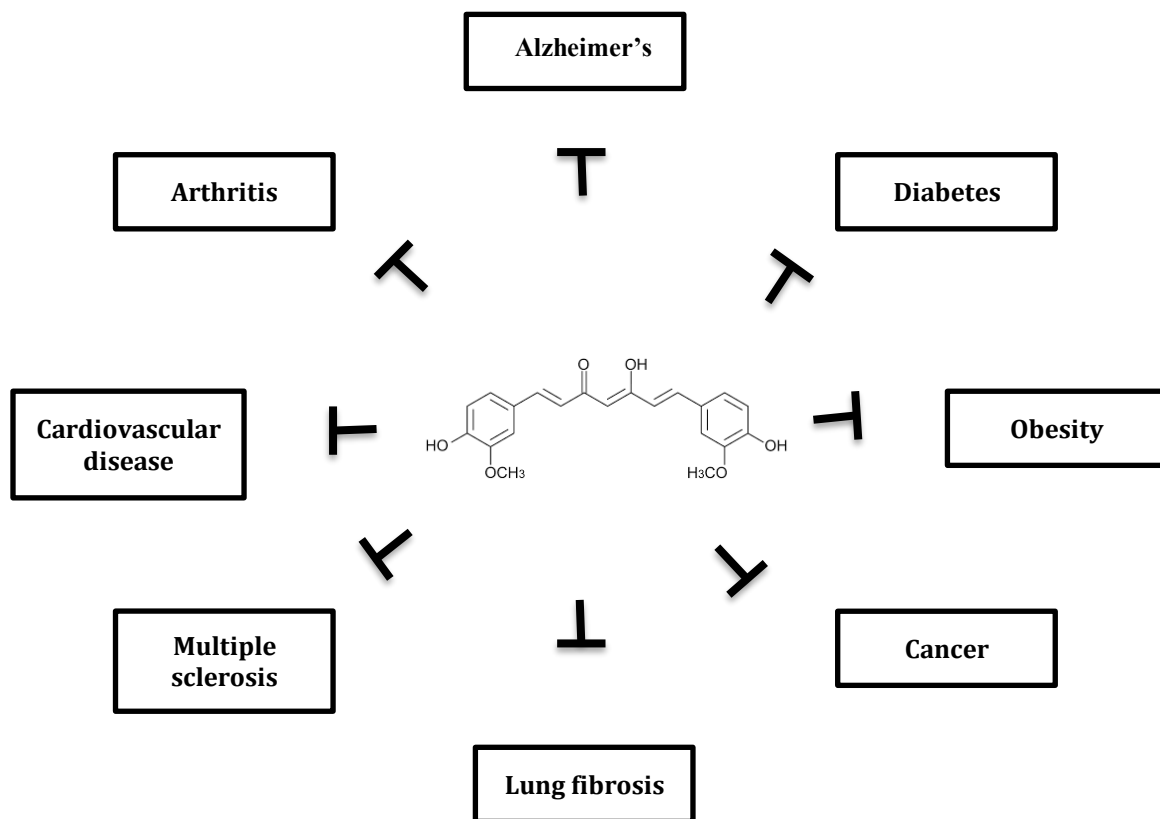
Fig 1.5: Proposed health benefits of curcumin. Curcumin has been implicated in having therapeutic capabilities and is used to treat a myriad of diseases.

studies aimed at understanding the fundamental molecular mechanisms controlling the action and cellular response to curcumin.

4. Studies on curcumin in other model systems

The absorption, metabolism and bioavailabity of curcumin have been studied in rodents. About 75% of a 1g/kg dose administered to rats through diet was excreted in feces with slight amounts also appearing in the urine (Wahlstrom and Blennow, 1978). In another study, after oral administration of 400mg of curcumin to rats, 60% was absorbed with no curcumin found in heart blood and a negligible amount found in portal blood (Ravindranath and Chandrasekhara, 1980). In a study, using ^{3}H-radiolabelled curcumin, there were detectable amounts of curcumin in blood, liver and kidney with doses ranging from 10 to 400 mg of curcumin per rat with the majority of the compound being excreted in feces, a third of it unchanged (Ravindranath and Chandrasekhara, 1981b). The bioavailabity of curcumin after oral administration has been compared to intraperitoneal (i.p.) administration in mice. After oral administration of 1g/kg of curcumin, low plasma levels of 0.13 μg/ml were observed after 15 minutes and higher plasma levels of 0.22 μg/ml observed after 1 hour. Plasma concentration levels were undetectable after 6 hours. Plasma levels of 2.25 μg/ml after i.p. administration were observed within 15 minutes and declined after 1 hour (Pan et al., 1999). Other studies have shown large quantities of curcumin and its metabolites in bile (Holder et al., 1978; Ravindranath and Chandrasekhara, 1981a). All these studies done in rodents show that curcumin exhibits a low systemic bioavailabity with large quantities being excreted in the bile. The major metabolites

of curcumin found in the bile are tetrahydrocurcumin (THC) and hexahydrocurcumin glucuronides (Holder et al., 1978; Ravindranath and Chandrasekhara, 1981a) (Fig 1.6). A study on the fate of i.p.-administered curcumin using mass spectrometry suggested that curcumin was first biotransformed to dihydrocurcumin and THC and then converted to monoglucuronide conjugates (Pan et al., 1999).

There is also a body of work done with curcumin in microorganisms and model eukaryotes. Cells with mutations in genes involved in iron and copper homeostasis in *Saccharomyces cerevisiae* are hypersensitive to curcumin with iron supplementation rescuing the phenotype (Minear et al., 2011). Another study demonstrated that yeast cells respond to curcumin treatment by phosphorylation of the mitogen-activated protein kinase (MAPK) Hog1, thereby activating the osmotic stress response mechanism in yeast (Azad et al., 2014). The effect of curcumin on ageing has been studied in *Drosophila melanogaster* (Shen et al., 2013; Zhang et al., 2015), *Caenorhabditis elegans* (Liao et al., 2011) and mouse (Nam et al., 2014) and shown to increase lifespan and reduce ROS, thereby suggesting that curcumin may be developed as a therapeutic agent in the treatment of age-related diseases.

C. Reactive Oxygen Species

I. Oxidative stress and oxidative damage

Reactive oxygen species (ROS) are oxygen-derived molecules including oxygen radicals such as superoxide ($O_2^{.-}$) and hydroxyl radical ($OH^.$), and non-radical derivatives such as hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$) (Fig 1.7).

A.

demethoxycurcumin

B.

bismethoxycurcumin

C.

tetrahydrocucumin

D.

hexahydrocurcumin

E.

octahydrocurcumin
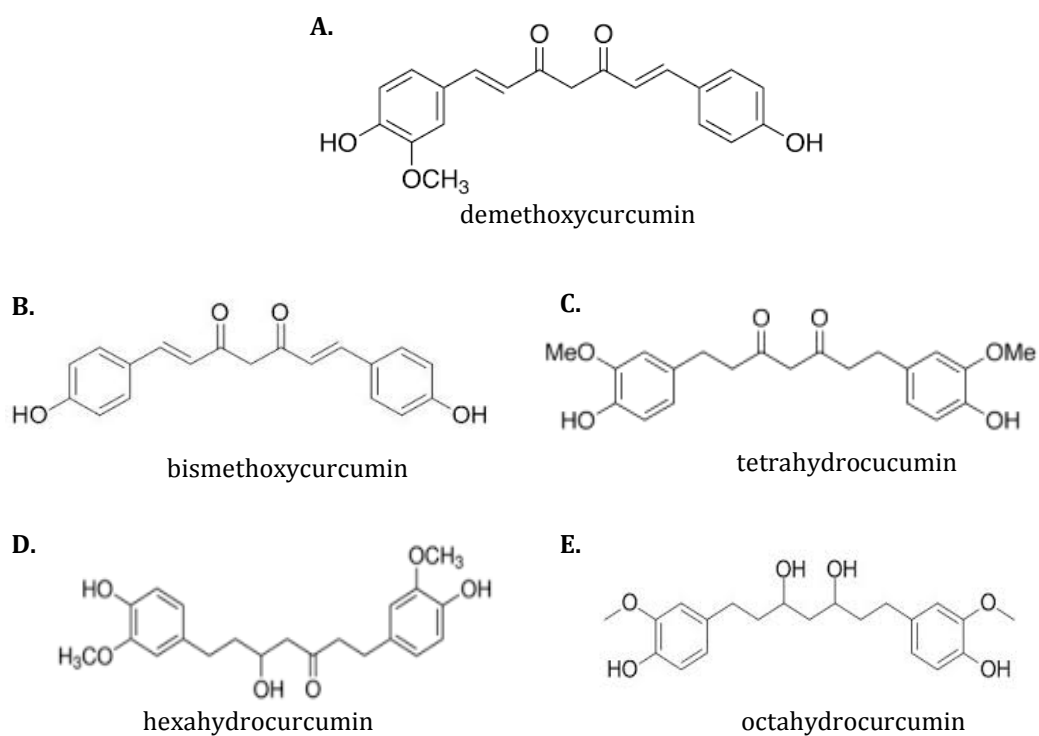
Fig 1.6: Curcumin and its metabolites. Studies done mostly in rodents have identified metabolites of curcumin generated either by conjugation or reduction as A) demethoxycurcumin B) bismethoxycurcumin C) tetrahydrocurcumin D) hexahydrocurcumin and E) octahydrocurcumin
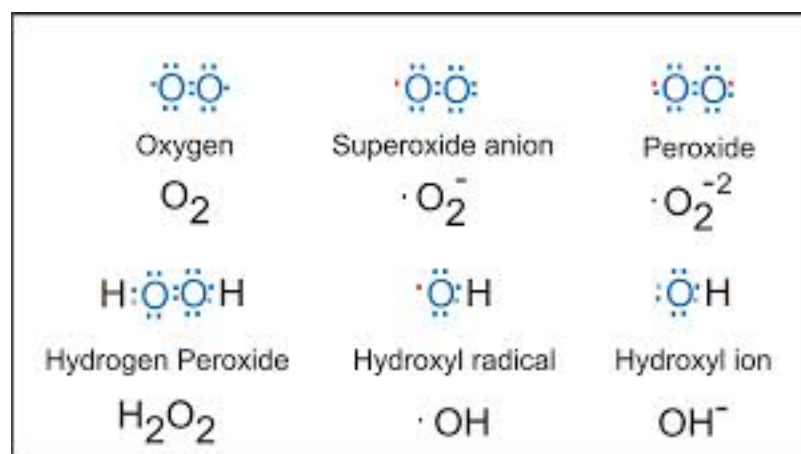
Fig 1.7: Chemical and Electron Structure of common reactive oxygen species.

Adapted from http://www.biotek.com/resources/articles/reactive-oxygen-species.html.

Accumulation of ROS can lead to oxidative stress and significant damage to cells by interacting with target molecules including proteins, lipids, carbohydrates and nucleic acids (Halliwell and Gutteridge, 1999; Harman, 1956). The cell has several ways of generating ROS. One way is through the use of a family of enzymes that depend on NADPH for their activity (Babior et al., 1975).

The NADPH oxidase enzymes include a seven member family, Nox 1-5 and Duox 1-2 that are evolutionary conserved and are essential in signal transduction mechanisms in the cell (Brown and Griendling, 2009). The NOX family of enzymes are transmembrane proteins that transport electrons across the plasma membrane and reduce oxygen to superoxide. The activities of other cytosolic enzymes such as cyclooxygenases, xanthine oxidase, lipoxygenases and cytochrome p450 enzymes also generate ROS within the cell. ROS generation can also occur as a byproduct of biological reactions including aerobic metabolism. In fact, the bulk of cellular ROS is generated within the mitochondria (Boveris and Cadenas, 1975; Boveris and Chance, 1973).

II. Antioxidant defenses

Because of the potentially damaging effects of ROS, cells use enzymes such as superoxide dismutase, peroxidase and catalase as antioxidant defense mechanisms (McCord et al., 1971) (Fig 1.8). Other non-enzymatic molecules capable of inhibiting ROS include vitamin E, uric acid and curcumin (Ames et al., 1981; Herrera and Barbas, 2001; Joe and Lokesh, 1994).
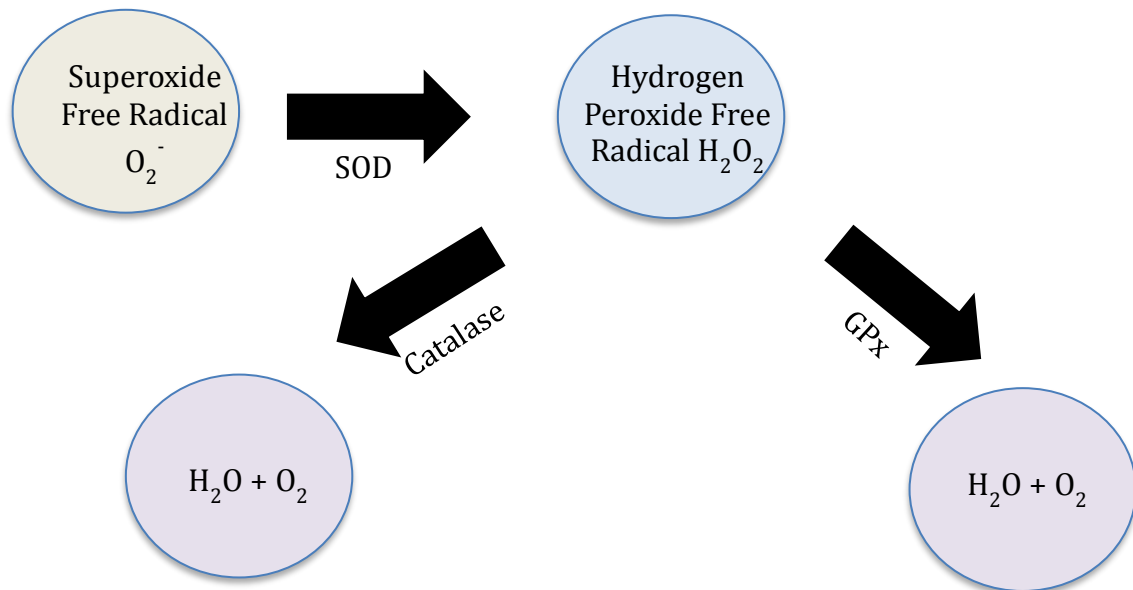
Fig 1.8: Antioxidant defense. The cell's first line of defence against antioxidants is the dismutation of the superoxide radical into hydrogen peroxide catalyzed by SOD. Catalase and glutathione peroxidase (GPx) then catalyze the decomposition of hydrogen peroxide to molecular oxygen and water.

Superoxide dismutase (SOD). A blue green copper-containing protein called haemocuprein was isolated from bovine blood in 1938. In 1978, a similar protein, hepatocuprein, was isolated from horse liver. Subsequently, similar proteins such as cerebrocuperin from brain and erythrocuperin, which contained zinc as well, were isolated (Markowitz et al., 1959). No enzymatic functions were attributed to these proteins until 1969. Erythrocuperin was shown to be able to remove $O_2^{.-}$ catalytically. Erythrocuperin became known as the copper and zinc containing superoxide dismutase (SOD) (McCord and Fridovich, 1969).

The family of SODs bind different metals to their active sites. The Cu/Zn form has copper and zinc as the metal cofactor. Cu/Zn SODs are present in all eukaryotes and mostly found in the cytosol of animal cells but Cu/Zn SODs are also present in lysosomes, the nucleus and the mitochondrial intermembrane space. Eukaryotic Cu/Zn SOD enzymes are made up of two protein subunits with each subunit containing a copper and zinc ion joined by hydrophobic and electrostatic forces, with a molecular mass of about 32,000 (Fridovich, 1995). Although rare, Cu/Zn SODs are also found in prokaryotes.

Catalase. Catalase is a ubiquitous enzyme found mostly in peroxisomes of nearly all aerobic organisms. It catalyzes the decomposition of hydrogen peroxide to water and oxygen, thereby protecting the cell against oxidative damage caused by peroxides. As a result of its scavenging properties, catalase has been used as a biomarker of stress in many species (den Besten et al., 2013; Kumari et al., 2014; Vlahogianni et al., 2007).

III. Antioxidant defense mechanisms in *D. discoideum*

Little is known about the cellular response to oxidative stress in *D. discoideum.* ROS can result from exposure to ultraviolet (UV) and has been shown to be involved in the induction of 8-Oxo-2' -deoxyguanosine by UV radiation (Zhang et al., 1997). *D. discoideum* is highly resistant to UV (Deering, 1988; Yu et al., 1998) and this has been partially attributed to the upregulation in nucleotide excision repair (NER) genes, *rep*B and *rep*D (Lee et al., 1997), and *rep*E (Alexander et al., 1996).

SOD in *D. discoideum*. *D. discoideum* encodes and expresses a number of SODs (Akaza et al., 2002; Garcia et al., 2000; Tsuji et al., 2002) corresponding to the SODs found in humans (Table 1.1).

The sequences for the *sod*A, *sod*B, (Tsuji et al., 2002) and *sod*C (Tsuji et al., 2003) genes suggest that they are Cu/Zn enzymes. The *sod*A and *sod*B proteins both contain charged amino acids and hydrophobic stretches in the N-termini suggesting that they are extracellular. They are expressed during both mitotic growth and multicellular development. One study showed that *sodA* was induced by H_2O_2 but not UV, while *sodB* was induced by both H_2O_2 and UV (Tsuji et al., 2002). In contrast another study showed that *sodA* was not induced by either H_2O_2 or UV (Garcia et al., 2000).

Studies on *D. discoideum sod*C showed that expression decreased in the presence of H_2O_2 and that *sod*C null cells were multinucleate although the meaning of this was not investigated (Tsuji et al., 2003) and that *sod*C regulates the small GTPase RAS. *D.*

D. discoideum gene	Human Orthologue	Reference
sodA	SOD1	(Garcia et al., 2000)
sodB	-	(Tsuji et al., 2002)
sodC	-	(Tsuji et al., 2003)
sodD	SOD1	(Akaza et al., 2002)
sodE	SOD1	Dictybase.org
sodF	SOD1	Dictybase.org
sod2	SOD2	Dictybase.org

Table 1.1: Superoxide dismutase (SOD) genes in *D. discoideum*. *D. discoideum* has seven SOD genes, four of which (*sod*A, D, E and F) share sequence homology with human SOD1.

*discoideum sod*C null cells have constitutively high activation of Ras, and *sod*C null cells treated with the scavenger XTT reduced the up-regulation of Ras (Veeranki et al., 2008).

*sod*D is 48% identical to the cytosolic Cu/Zn SOD of *S. cerevisiae* and is only expressed late in development (18 hours) and is not expressed in a mutant which is unable to make spores. *sod*D null cells grow and develop normally, but mutant spores were more sensitive to UV light compared to wild-type spores (Akaza et al., 2002).

In another study, it was shown that superoxide is generated as a signaling molecule in *D. discoideum* early in development in response to secreted factors that are required to initiate development (Bloomfield and Pears, 2003). Scavenging superoxide pharmacologically or by overexpressing *sod*A inhibits cell aggregation and further development. These data suggest that ROS signaling arose early in the evolution of multicellular development, and that synthesis of superoxide is essential for morphogenesis.

Catalase. Catalase is a ubiquitous enzyme found mostly in peroxisomes of nearly all aerobic organisms. It catalyzes the decomposition of hydrogen peroxide to water and oxygen, thereby protecting the cell against oxidative damage caused by peroxides. As a result of its scavenging properties, catalase has been used as a biomarker of stress in many species (den Besten et al., 2013; Kumari et al., 2014; Vlahogianni et al., 2007).

Catalase in *D. discoideum*. Catalase is present in *D. discoideum* and its activity in *D. discoideum* has been shown to be higher than in other organisms possibly due to the amount of stress generated as a result of their natural habitat in soil (Madigan and Katz, 1989). *D. discoideum* encodes two catalase genes (*cat*A and *cat*B) which are similar to catalase genes found in humans and other organisms, and are differentially expressed during growth and development (Garcia et al., 2000).

*cat*A is expressed uniformly throughout growth and development whereas *cat*B is only expressed in the final stages of development. Subcellular fractionation studies showed that CatA enzyme activity is found only in prestalk cells whereas CatB enzyme activity is found in prespore cells only, suggesting that the CatB enzyme protects spores from oxidative damage (Garcia et al., 2000). H_2O_2 does not induce expression of *cat*A in *D. discoideum* (Garcia et al., 2000), however *cat*A and *cat*B null mutants show increased sensitivity to oxidative stress (Garcia et al., 2003; Garcia et al., 2000). *D. discoideum* mutants with disruptions in the *cat*A gene grow and develop at wild-type rates. Remarkably, *cat*A mutants show cross-resistance to methanol, acriflavine and thiabendazole (Garcia et al., 2002) but the mechanism of this cross resistance is unknown.

yakA in *D. discoideum*. The protein kinase *yak*A, similar to Yak1p in yeast mediates the transition to development upon starvation. *yak*A null mutants fail to aggregate and expression of genes encoding adenylyl cyclase, cAMP receptors and *pka*C, which encodes the catalytic subunit of PKA, are significantly reduced (Souza et al., 1998).

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*Yak*A null cells are also hypersensitive to oxidative and nitrosative stress. Sodium nitroprusside and H_2O_2 hypersensitivity in *yak*A null cells is suppressed in a *yak*A/*kea*A null background, establishing a role for *kea*A in the stress response of *D. discoideum* (Mantzouranis et al., 2010).

<u>*kea*A in *D. discoideum.*</u> *kea*A is a member of the kelch-domain superfamily of proteins (Adams et al., 2000; Xue and Cooley, 1993). Kelch or kelch-like proteins are characterized by the presence of a kelch motif of 44-56 amino acids usually repeated 5-7 times. Each motif forms a β propeller tertiary structure (Ito et al., 1994). Kelch proteins have been isolated in many organisms including human, mouse and rat, *S. cerevisiae, Drosophila* and *C. elegans*. Members of the kelch-domain superfamily of proteins function mainly in actin-cytoskeleton and protein-to-protein interactions (Adams et al., 2000). *D. discoideum kea*A is similar to the mouse and rat kelch protein 8, the Drosophila Diablo protein and the human Keap1 protein.

Keap1 is a key regulator of the antioxidant response mechanism in human cells. Keap1 binds and sequesters Nrf2 in the cytoplasm. Keap1-bound Nrf2 is eventually targeted for ubiquitin-mediated degradation. Upon oxidative stress and keap1 oxidation, Nrf2 is released into the nucleus where it binds to antioxidant response element sequence-containing genes, inducing the activation of antioxidant response genes. We know that *D. discoideum kea*A plays a role in stress survival, however the mechanism is unknown. A yeast 2 hybrid system to investigate possible KeaA interactions showed no interaction with other proteins (Mantzouranis et al., 2010).

D. Summary

The efficacy, safety and mechanism of action of botanicals is an important area of biomedical investigation that requires an intensified research effort. This field of investigation overlaps with the large research efforts on cellular signaling and the control of reactive oxygen species in cells. Curcumin is widely used worldwide for the treatment of many diseases and symptoms. Numerous molecules and molecular pathways have been implicated in the cellular response to curcumin, but no consistent picture has developed that provides a useful understanding of whether curcumin has beneficial pharmacological effects and what are the underlying mechanisms.

Experimental systems allowing facile biochemical and genetic studies have previously proven to be key to deciphering many complex biological problems. *D. discoideum* affords this mix of experimental attributes and has previously been used to make unique discoveries on the mechanism of action of drugs of importance to human health. This same system has now been used to provide novel insights on the effect of curcumin on cellular physiology. This new conceptual framework can now be translated and validated in human cells.

2. The botanical curcumin negatively regulates transcription of *Dictyostelium discoideum* antioxidant genes and increases reactive oxygen species via a PKA dependent mechanism

A. Abstract

Botanicals are widely used as dietary supplements and for the prevention and treatment of disease. Despite a long history of use, there is generally little evidence supporting the efficacy and safety of these preparations. Curcumin has been used to treat a myriad of human diseases and is widely advertised and marketed for its ability to improve health, but there is no clear understanding how curcumin interacts with cells and effects cell physiology. *D. discoideum* is a simple eukaryotic lead system that allows both facile genetic and biochemical studies. These studies show that curcumin has a novel effect on gene transcription and cell proliferation. The effect is complex and involves an early transient effect on the transcription of approximately 600 genes. This is followed by later unique transcriptional changes and a protein kinase A dependent decrease in catalase A and three superoxide dismutase enzymes. Although this results in an increase in reactive oxygen species (ROS), the effects of curcumin do not appear to be the result of oxidation. The study underscores our lack of understanding associated with the prophylactic and therapeutic use of curcumin and other xenobiotics.

B. Introduction

The use of botanicals as dietary supplements is becoming increasingly popular. According to the World Health Organization (WHO), it is estimated that 80% of the world's population uses botanicals as part of their primary health care. In the United States, 20% of Americans use botanicals, with billions of dollars spent each year on these natural remedies (Bent, 2008). The global botanical market was valued at $54.6 billion dollars in 2013 with a forecasted market value estimated to reach $90.2 billion by 2020 (Research and Markets, 2015).

Curcumin is the active ingredient in turmeric (Huang and Beevers, 2011) and has been widely used in traditional medicine, especially in India, China and Thailand. It has been used to treat many diseases including anorexia, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis (Jayaprakasha et al., 2006). Curcumin has been linked to a wide spectrum of pharmacological effects including anti-carcinogenic, anti-inflammatory, Alzheimer's prevention and antioxidant activity (Sharma et al., 2005). Curcumin has been further implicated in controlling the growth, development and spread of cancer by interfering with the cell cycle, apoptosis, proliferation, angiogenesis and metastasis (Anand et al., 2008; Conney et al., 1991).

Chronic low-level inflammation has been associated with many diseases including heart disease (Libby, 2002), metabolic syndrome (Lumeng and Saltiel, 2011) and cancer (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Some studies have

implicated curcumin in blocking NF-$_k$B, a transcription factor that plays a key role in turning on genes related to inflammation (Singh and Aggarwal, 1995). Indeed, studies have indicated that curcumin has higher efficacy and fewer side effects compared to other anti-inflammatory drugs (Lal et al., 1999; Takada et al., 2004). Alzheimer's disease, the most common neurodegenerative disease in the world, is characterized by a buildup of protein tangles in the brain known as amyloid plaques, and studies have shown curcumin to have a potential role in the prevention of the disease (Mishra and Palanivelu, 2008) and even the ability to clear amyloid plaques by enhancing the surface binding of amyloid-beta to macrophages and intracellular phagocytosis leading to amyloid-beta degradation (Zhang et al., 2006).

An antioxidant role is often ascribed to curcumin. Oxidative damage, the reaction of free radicals with cellular lipids, proteins and DNA, is associated with the cause of many diseases and ageing. ROS such as superoxide anions, peroxides and hydroxyl radicals are by-products of aerobic metabolism and their accumulation can lead to oxidative stress and significant damage to cells (Halliwell and Gutteridge, 1999; Harman, 1956).

Because of the potentially damaging effects of ROS, cells use enzymes such as superoxide dismutase, peroxidase and catalase as antioxidant defense mechanisms. (McCord et al., 1971). Other non-enzymatic molecules capable of inhibiting ROS include vitamin E and uric acid (Ames et al., 1981; Herrera and Barbas, 2001; Joe and Lokesh, 1994). Curcumin has been reported to have antioxidant effects (Kunchandy

and Rao, 1990) in rat peritoneal macrophages (Joe and Lokesh, 1994) and in red blood cells (Tcnnesen and Greenhill, 1992).

Despite this large and growing interest in the use of botanicals in disease treatment and prevention, there is little evidence regarding their efficacy, safety and long-term effects. Importantly, the fundamental mechanisms associated with the cellular response to botanicals are generally not clearly understood, and there are often unknown off-target effects. This is supported by the bafflingly large number of effects associated with curcumin. Although evidence suggests that curcumin is an antioxidant, recent reports suggest that curcumin has pro-oxidant effects increasing ROS and thereby inducing oxidative stress within cells (Khan et al., 2012; Yoshino et al., 2004). Possessing both antioxidant and pro-oxidant effects confounds the rational therapeutic use of curcumin. Thus, a better understanding of the molecular mechanisms underlying the response to curcumin is required to validate its pharmacological use in the treatment of diseases and would benefit from a genetically tractable model system that would be useful to interrogate the molecular effects of curcumin on cell physiology.

The social amoeba, *D. discoideum*, has proven to be an excellent model for the molecular genetic study of the mechanisms of action of drugs and their effects on the cell (Alexander et al., 2006; Williams et al., 2006). *D. discoideum* is a haploid eukaryote which proliferates rapidly as single cells. Its cell structure and physiology are similar to mammalian cells and has been intensely studied (Kessin, 2001).

Moreover, the results from this lead genetic system have been readily validated and translated to human cells (Alexander and Alexander, 2011; Ludtmann et al., 2011). Therefore, *D. discoideum* offers a useful lead genetic system with which to study the underlying mechanisms of action of curcumin. Indeed, a recent study has investigated the effect of curcumin on multicellular development in *D. discoideum* and showed down-regulation of a number of early developmentally regulated genes (Garige and Walters, 2015).

In contrast, the current study is focused on the effect of curcumin on cell viability and cell proliferation. The study shows that curcumin has a profound pleiotropic effect on the cell physiology of mitotically dividing *D. discoideum* cells, including an accumulation of ROS which is regulated by down-regulation of antioxidant gene expression. This transcriptionally regulated physiological response was shown to be mediated, in part, by protein kinase A (PKA). Moreover, RNA sequencing (RNA-seq) analysis reveals an even broader transcriptional response elicited by curcumin and suggests a variety of previously unknown molecular pathways that respond to curcumin.

Taken together, these studies highlight the relative lack of understanding about the effect of curcumin (and by extension, other botanicals) on genetic regulation and cell physiology. Importantly, they provide new avenues of investigation that can be further interrogated using the genetic tools of *D. discoideum* and then translated in

human cells as has been previously done in studies on other medically important drugs (Alexander and Alexander, 2011; Ludtmann et al., 2011).

C. Materials and Methods

I. Reagents.

Chemicals were from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Curcumin stock solutions were freshly prepared in DMSO and the concentration was determined by measuring the absorbance at 423 nm, using the empirically determined extinction coefficient $\varepsilon = 54954$ cm^{-1} M^{-1}. The stock solutions were then adjusted to 50 mg/ml (Table 2.1), and stored frozen at $^-20°$C. Sub-stock solutions were made by dilution in DMSO so that curcumin was always delivered in the same volume yielding a final DMSO concentration of 0.2% which has no effect on *D. discoideum* cell proliferation.

Tert-butylhydroquinone (TBHQ) and N-acetylcysteine (NAC) were prepared as a stock solution of 5 mg/ml and 100 mg/ml in DMSO or water, respectively.

II. Strains and conditions for growth.

The *D. discoideum* strains used in this study are listed in Table 2.2 along with their genotypes, phenotypes and sources. The cells were grown axenically at 22°C in shaken HL-5 medium (Sussman, 1987). New cultures were started monthly from desiccated spores. Cell number was monitored by multiple counts in a hemocytometer. Stock cells were always kept in log-phase growth and never allowed

Curcumin 50 mg/ml ➡ 1:100 dilution ➡ 1:100 dilution

Wavelength (nm)	Absorption
587	0.338
423	0.402
257.5	0.206

Table 2.1: Spectral properties of curcumin in dimethyl sulfoxide (DMSO). 50 mg/ml curcumin stock was diluted $1:10^4$. Shown are the absorption peaks of curcumin. Curcumin stock solutions are standardized to this absorption.

NAME	GENOTYPE	PHENOTYPE	MUTAGENESIS METHOD	PARENT	REFERENCES
RegA	*reg*AΔ	Aberrant chemotaxis to cAMP, aberrant fruiting body morphology, cisplatin resistant	Homologous recombination	Ax4	(Shaulsky et al., 1998)
ErkB	*erk*BΔ	Abolished aggregation	Homologous recombination	Ax4	(Segall et al., 1995)
RegA/ErkB	*reg*AΔ/*erk*BΔ	Abolished aggregation	Homologous recombination	*erk*B-	(Maeda et al., 2004)
DBS0266778 (IR41)	*cat*AΔ	Abolished catalase A activity	Homologous recombination	Ax4	(Garcia et al., 2002)
DBS0237087	*yak*AΔ	Abolished aggregation	Homologous recombination	Ax4	(van Es et al., 2001)
DBS0237194	*sod*A OE	Abolished aggregation, overexpressed SOD	Random insertion	Ax2	(Bloomfield and Pears, 2003)
DBS0235411	*pka*RΔ	Abolished aggregation	Homologous Recombination	Ax4	(Shaulsky et al., 1998)
DBS0236783	*pka*CΔ	Abolished aggregation, abolished PKA catalytic activity	Homologous recombination	JH10 (Ax4)	(Mann and Firtel, 1991)
DBS0235418 (4M)	*pka*R OE	Neomycin resistant, PKA regulatory subunit overexpressor, axenic, abolished aggregation	Random insertion	Ax2	(Simon et al., 1989)

Table 2.2: *D. discoideum* **strains used in this study.** Δ = Null gene, OE = overexpressed gene, Ax4/Ax2 = WT.

to grow beyond 5×10^6 cells/ml. Logarithmically growing cells at a density of $2\text{-}4 \times 10^6$ cells/ml in HL-5 medium were used for experiments and had a generation time of 10-13 hours. Curcumin and other drugs were added directly to the cell cultures by dilution from stock solutions (see Table 2.3) at the indicated final concentrations, and all cultures (experimental and control) received the same concentration of vehicle. The final DMSO concentration was 0.2%, which had been shown to have no effect on cell proliferation when compared to untreated controls.

III. Measurement of cell proliferation and viability.

Cell proliferation measurements employed a luminescent cell viability method used to determine the number of viable cells based on the amount of ATP in metabolically active cells (Min et al., 2006). For each curcumin concentration to be tested, the parent culture ($2\text{-}4 \times 10^6$ cells/ml) was diluted to 10^5 cells/ml in 5 ml aliquots, after which compounds or vehicle was added. All experiments were done in triplicate. The CellTiter-Glo® luminescent cell viability assay (Promega Cat. No. G7571) was performed in opaque 96 well plates (Thermo Scientific Cat. No. 4912). At the indicated times, 100 μl of treated or control cells were sampled and mixed with an equal volume of CellTiter-Glo® for 10 minutes at 22°C. Luminescence was measured using a microplate luminometer (Veritas). The data represent the average of three replicates, and were always in the linear range of the technique (Min et al., 2006).

Curcumin stock (mg/ml)	Dilution	Stock concentration after dilution (mg/ml)	Final concentration 1:500 (µg/ml)
50	1:40	1.25	2.5
50	1:20	2.5	5
50	1:10	3.75	7.5
50	1:10	5	10
50	1:8	6.25	12.5
50	1:6.66	7.5	15

Table 2.3: Curcumin working concentrations. 50 mg/ml curcumin stock was diluted so that equal volume (10 µl) of diluted stock could be added to medium (5 ml) to obtain desired final concentration of curcumin and a final vehicle concentration (DMSO) of 0.2%.

Clonal plating was performed on SM plates in association with *Klebsiella aerogenes*. Multiple plating at different dilutions was performed to ensure that the counts were in the linear range of this technique.

IV. Catalase Activity Assay.

Catalase activity was determined as described (Garcia et al., 2000). Dividing cells in axenic medium (10 ml in 125 ml flasks) were treated with compounds at the indicated concentration, harvested and washed by centrifugation in SS buffer (10 mM NaCl, 10 mM KCl, 2.7 mM $CaCl_2$). The cells were pelleted and then lysed in 100 μl lysis buffer (10 mM potassium phosphate, pH 7.0, 0.1% Triton X-100, 1X protease inhibitor) for 5 minutes on ice. Samples of 5μl and 10 μl of the cell lysate were assayed for catalase activity by mixing with 10 mM H_2O_2 in 50 mM potassium sulfate, pH 7.0 in a final volume of 1 ml. Reduction of absorbance at 240 nm was monitored over a two-minute period at 10 second intervals with a spectrophotometer. (Garcia et al., 2000). Boiling eliminates all of the activity of the extracts. One absorbance unit equals 1 μmole of H_2O_2 using an extinction coefficient of 43.6 M^-cm^-. Multiple assays were performed on multiple biological samples. Protein was measured using the Bradford assay (Pierce) adjusting the standard curve for the final concentration of lysis buffer added along with the unknown samples. Specific catalase activity was expressed as μmole/min/mg protein.

V. Superoxide Dismutase Activity Assay.

This assay employs hematoxylin which, above pH = 7, generates superoxide (SO) and is in turn oxidized by the SO. The result is a concentration dependent increase in absorbance with a spectrum between 400 and 670 nm. Superoxide dismutase (SOD) inhibits this increase in absorbance in a concentration dependent manner, and the enzyme activity is expressed as the percent of inhibition of the reaction without SOD.

Hematoxylin is dissolved in 0.05 M mono-potassium phosphate and stored in aliquots frozen at -80°C to ensure that every assay is using the identical substrate. The reaction is started by adding 30 μl of the hematoxylin solution to 970 μl of 0.05 M potassium phosphate buffer pH 7.6 and reading the absorbance increase at 560 nm. Curcumin does not absorb at this wavelength. This ratio generates an absorbance of 0.066 per minute and which allows the sensitivity necessary to detect SOD quantitatively.

Cells were harvested, washed in SS buffer, and pellets of 10^7 cell were frozen at -80°C. Freshly prepared cold lysis buffer (0.05 M potassium phosphate buffer pH 7.6, 0.1% Triton X100, and 1x protease inhibitor cocktail) was added to the frozen pellets and vortexed to lyse the cells. Dilutions were made with lysis buffer and samples of the dilutions were assayed for their ability to inhibit the reaction by the catalytic degradation of SO. The lysis buffer had no effect on the reaction. Multiple assays are done on multiple biological samples. Protein was measured using the Bradford assay

(Pierce) adjusting the standard curve for the final concentration of lysis buffer added along with the unknown samples.

Boiled samples for each dilutions are assayed as background because there is an unidentified heat stable material in the lysates that inhibits some of the reaction. This material could not be removed by dialysis.

VI. Superoxide determination.

Superoxide production was measured utilizing an assay based on the reduction of the tetrazolium dye XTT (2,3-Bis- (2-Methoxy-4-Nitro-5-Sulfophenyl)- 2H-Tetrazolium-5-Carboxanilide (Able et al., 1998). XTT was purchased from Molecular Probes (Eugene, Oregon) and stock solutions (10 mM) were prepared in 20 mM potassium phosphate buffer, pH 7. XTT was added to growing cells ($2-4 \times 10^6$ cells/ml) at a final concentration of 500 μM and incubated for the indicated times. Reaction with superoxide converts the pale yellow dye to a bright orange colored water soluble formazan product which can be measured by spectrophotometry at 470 nm (Bloomfield and Pears, 2003; Ukeda et al., 1997). One ml aliquots of treated cells were harvested, centrifuged and the supernatant measured in a spectrophotometer. Superoxide production was expressed as absorbance at 470 nm per 10^8 cells. Controls were run so that the absorbance of curcumin at 470 nm could be subtracted from the experimental samples.

VII. Hydrogen Peroxide determination

Hydrogen peroxide (H_2O_2) was detected using an assay based on amplex red oxidation. The Amplex Red hydrogen peroxide/peroxidase assay kit was purchased from Molecular Probes (Eugene, OR). In this assay the Amplex Red reagent, in the presence of horseradish peroxidase (HRP), produces a red oxidation product upon reaction with H_2O_2. The oxidation product, resorufin, can be measured by spectrophotometry at 540nm. Stock solutions were prepared according to the manufacturer's instructions. A working solution of 100 μM Amplex Red reagent was prepared by mixing 100 μl of Amplex Red stock solution, 200 μl HRP stock and 9.7 ml HL5 medium. 50 μl of the Amplex Red working solution was transferred to microplate wells.

To prepare the samples, cells were grown to $1\text{-}2\times10^6$ cells/ml. Curcumin or vehicle was added to the cell cultures so that the concentrations were twice the desired final concentration. Immediately, 50 μl of the cells containing curcumin or vehicle was added to microplate wells containing 50 μl of Amplex Red working solution to bring final curcumin concentration to 1X. The plates were shaken at 200 RPM at 22°C and read at the indicated time points to measure the accumulation of H_2O_2. Controls were run so that any absorbance of curcumin at 540nm could be subtracted from the experimental samples. The measurements were done in quadruplicate.

VIII. qRT-PCR.

Total RNA was prepared from 5×10^6 cells using Qiagen's RNEasy mini kit. RT-PCR was performed using the High Capacity cDNA Reverse Transcription kit purchased from Applied Biosystems (Foster City, California) following the manufacturer's instructions. A cocktail of random primers (included with kit) was used to amplify the first strand cDNA, which then served as template for subsequent PCR. The oligonucleotides used for the study of oxidative stress response enzymes are listed in Table 2.4.

IX. REMI Insertional Mutagenesis.

Ax4 parent cells were grown to a density of 2×10^6 cells/ml in liquid HL-5 medium and supplemented with 0.6 μg/ml vitamin B12 and 0.2 μg/ml folic acid. Four independent transformations, each at a cell density of 1×10^7 cells were performed. Cells were mutagenized by electroporation with pBSR1 plasmid which contains the gene for blasticidin S resistance (BSR) (Shaulsky et al., 1996), using established methods (Kuspa and Loomis, 1992). Transformants were selected for growth in HL-5 medium containing 10 μg/ml blasticidin purchased from Invivogen (San Diego, CA). These transformants were then subsequently selected for growth on plates containing 15 μg/ml curcumin. Surviving cells were plated and retested for growth in the presence of 15 μg/ml curcumin.

Gene	Forward primer	Reverse Primer	Size
IG7	5'-CTC GAT GTC GGC TTA ACA CA-3'	5'-AGG GAC CAA ACT GTC TCA CG-3'	109
*cat*A	5'-GCT GCT CGT CAA CCA TAC AA-3'	5'-CGG ACA TGT GAC CGA CTA AA -3'	121
*sod*A	5'-GGT GAA CAC ACA ATC GTT GG-3'	5'-CCA ATG ACA CCA CAA CCA AG -3'	249
*sod*B	5'-AAC CAC TGG CAA TGC AAA TA-3'	5'-CAA TAG CGG CAG ATG GAG AT -3'	110
*sod*E	5'-TGG GTG TTT ATC AGC AGG AG-3'	5'-CCT AAA TCA CCA ACA TGA CGA -3'	90
*sod*2	5'-GGT CCA TTG GCT GAT GCT AT-3'	5'-TAA CCA ACC CCA ACC AGA AC -3'	182

Table 2.4 Primers used for qRT-PCR in this study.

To isolate the disrupted genes, genomic DNA from curcumin resistant mutants was digested with EcoRI (there are no EcoRI restriction sites in pBSR1 vector) to rescue the plasmid and the flanking genomic sequences were cloned into DH5α *E. coli*.

X. Transcriptional Analysis.

To determine the global transcriptional response to increasing concentrations of curcumin, wild-type cells (2×10^6 cells/ml) were grown in 20 ml of shaking culture in the presence of the indicated concentration of curcumin. Fifteen ml of the cultures were harvested at the indicated times. RNA was isolated using Ambion Trizol Reagent purchased from Life Technologies (Carlsbad, CA) according to the manufacturer's instructions. The samples were reverse transcribed, amplified and sequenced in the Baylor College of Medicine sequencing facility as described (Miranda et al., 2013).

D. Results

I. Curcumin reduces cell proliferation and viability.

In order to investigate the biochemical effects of curcumin on *D. discoideum*, we first determined its effect on cell proliferation by counting cells in the treated cultures. Fig. 2.1 A shows the results of treating axenically growing parent Ax4 cells with different concentrations of curcumin over a period of 4 days. The data show that curcumin inhibits the rate of cell proliferation in a dose dependent manner where 2.5
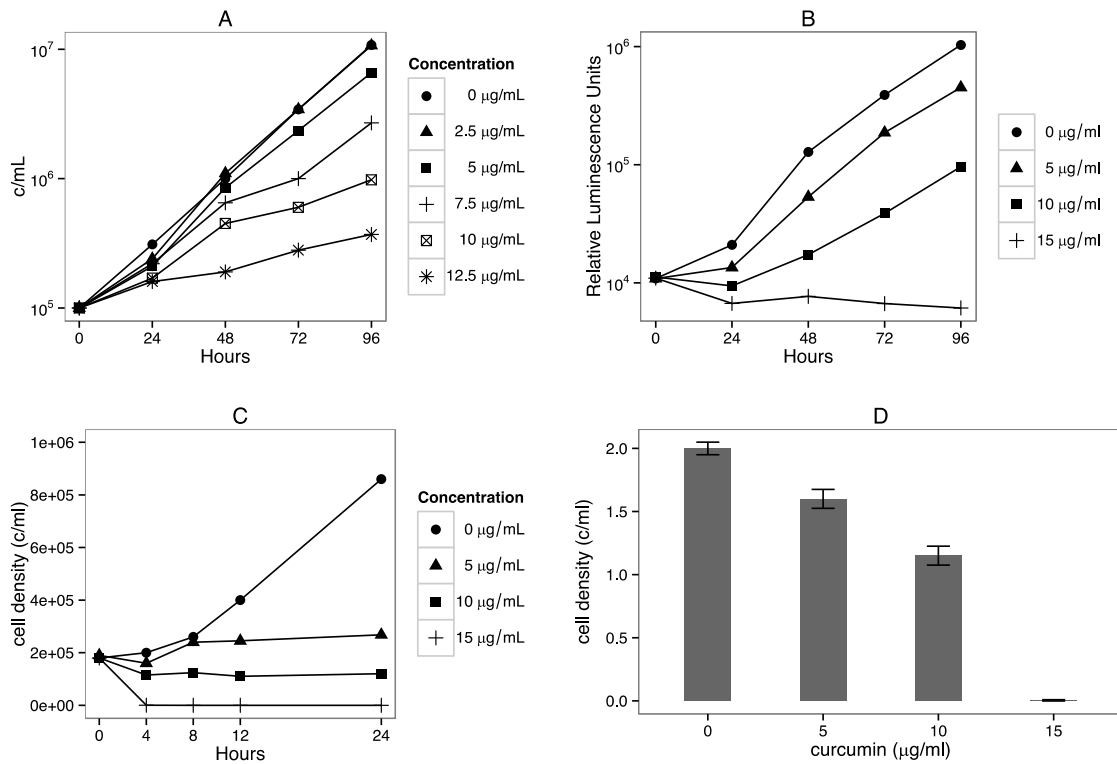
Fig. 2.1: Curcumin reduces proliferation and cell viability A) Axenically growing Ax4 cells were treated with curcumin at the indicated concentrations and cell density was monitored over four days by direct counting. B) In a separate experiment, viability of curcumin treated cells was assayed by measuring ATP in metabolically active cells using Promega's CellTiter-Glo®. C) Cell survival of curcumin treated cells was also determined by clonally plating cells on agar plates in association with *Klebsiella aerogenes.* D) The 4-hour time point from C) is presented in more detail.

μg/ml has no effect compared to the control and 12.5 μg/ml almost completely inhibits cell proliferation. Note that the data are plotted on a log scale.

We previously described a rapid and sensitive cell viability assay for *D. discoideum* using CellTiter-Glo® (Promega) that measures ATP in living cells (Min et al., 2006). Using this assay the experiment in Fig. 2.1 A was repeated [including higher curcumin concentrations (15 and 20 μg/ml)], and the data on rate of cell proliferation (Fig. 2.1B) are virtually identical to those obtained by direct cell counting. Notably, 15 μg/ml curcumin inhibits all cell proliferation over 4 days.

Taken together, the data in Fig. 2.1 A and B (which is repeated as a control in Figs 2.9) indicate that curcumin has a lasting inhibitory effect on cell proliferation over 4 days. However, the experiments do not address the question of whether curcumin has a cytotoxic effect rather than a cytostatic effect on the cells. To address this later point, axenically growing Ax4 cells were treated with curcumin and samples were taken at 0, 4, 8, 12, and 24 hours. The samples were then diluted and plated clonally to determine if there was any immediate cytotoxicity. As seen in Fig. 2.1 C and Fig. 2.1 D, where the 4-hour time point is presented in more detail, curcumin has an initial dose dependent toxicity on cells in addition to the persistent inhibition of cell proliferation seen in Figs. 2.1 A and B.

The data raised the possibility that curcumin was unstable in the growth medium. To test this, multiple flasks of growth medium were inoculated with 10 μg/ml curcumin

and at daily intervals cells were added at the same initial density of 1×10^5 cells/ml. As seen in Fig. 2.1 E the curcumin had the same effect on cell proliferation irrespective of how long it was in solution. Thus, curcumin stability is not a factor in these experiments.

II. Curcumin reduces catalase A and SOD enzyme activities

The experiments described above demonstrate a clear effect of curcumin on cell viability and proliferation. The effects of curcumin have been often attributed to antioxidant properties (Joe and Lokesh, 1994; Kunchandy and Rao, 1990; Tonnesen and Karlsen, 1985). The levels of anti-oxidant enzymes such as catalase and superoxide dismutase are often used as indicators (surrogates) for the level of ROS in cells (Mates, 2000; Weydert and Cullen, 2010). Therefore, we initially measured the effect of curcumin on these enzymes.

Cells treated with curcumin were assayed for the level of catalase A enzyme activity. Catalase A is the only catalase enzyme in proliferating cells (Garcia et al., 2000), although a second enzyme – catalase B – is expressed at a late stage of multicellular development (Garcia et al., 2003). The data demonstrate that the specific activity of catalase A in the presence of curcumin decreases to 50% in a dose (Fig. 2.2 A) and time (Fig. 2.2 B) dependent manner that is consistent with the inhibition of cell proliferation (Fig. 2.1). Further *in vitro* experiments showed that this loss of catalase A specific activity in the presence of curcumin was not due to a direct effect of
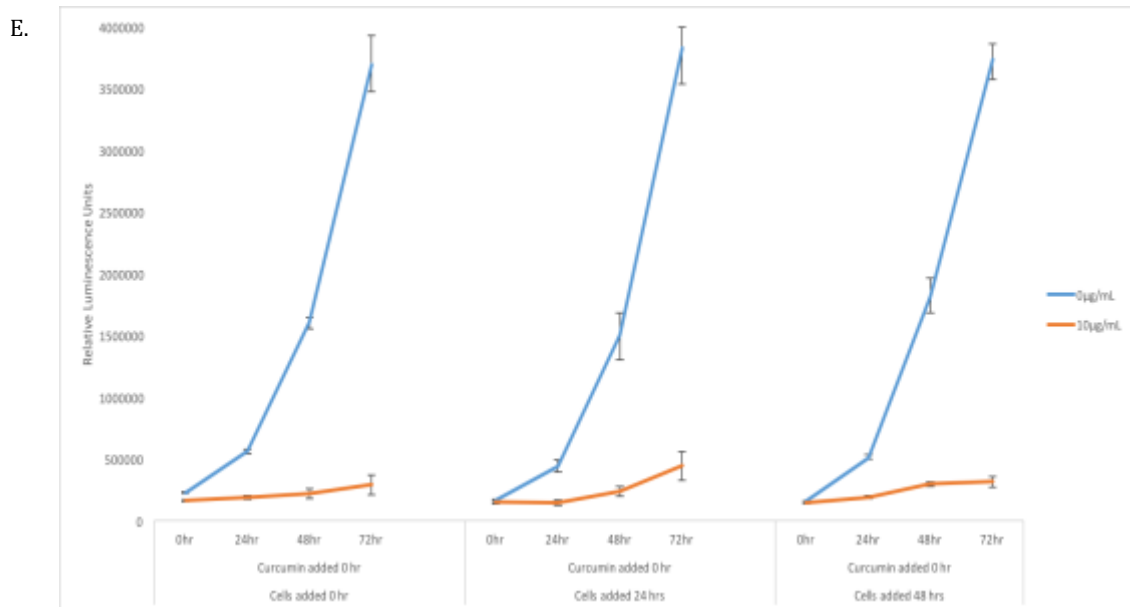
Fig. 2.1 cont'd: Curcumin reduces proliferation and cell viability. E) Curcumin

stability in HL5 growth medium was determined by adding curcumin to medium and

inoculating cells at time zero, 24 hours and 48 hours. Taken together, these results

show that curcumin has a lasting inhibitory effect on cell proliferation.
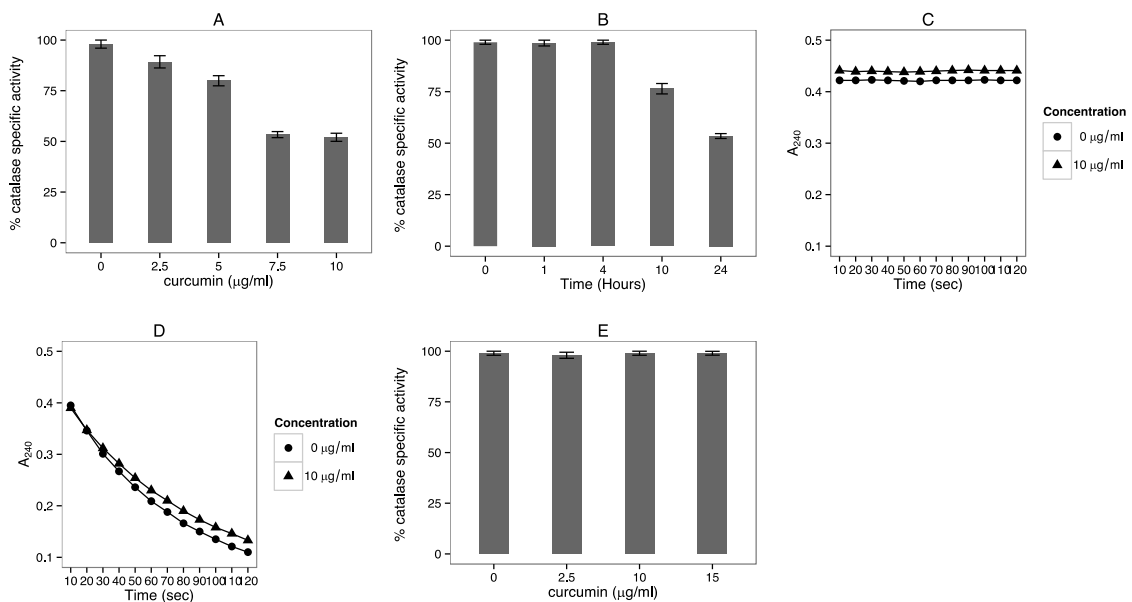
Fig. 2.2: Curcumin reduces catalase A enzyme levels but has no effect on enzyme catalysis A) Catalase A enzyme specific activity is reduced in curcumin treated cells in a dose dependent manner; B) Reduction in catalase A specific activity is not immediate and takes up to 24h to manifest itself indicating that it is not due to enzyme inhibition; C) Curcumin does not have an effect on the stability of H_2O_2; D) Curcumin by itself does not have a direct effect on the rate of catalase A activity; and E) Curcumin does not have an effect on the extent of catalase activity.

curcumin on the catalase A enzyme. Curcumin had no effect on the stability of H_2O_2 (Fig. 2.2 C), and neither the kinetics (Fig. 2.2 D) nor the extent (Fig. 2.2 E) of catalase A catalysis was effected by curcumin. These experiments (Fig. 2.2 C-E) show that the effect of curcumin on catalase A activity is not immediate and not at the level of enzyme inhibition.

Superoxide dismutase (SOD) was also assayed in cells treated with curcumin. The assay relies on the inhibition of superoxide accumulation by the action of SOD. In addition, the assay is presumably measuring the activity of all the SODs expressed in the cells. This is the first characterization of SOD enzyme activity in *D. discoideum*. Fig. 2.3A shows that, similar to the catalase A activity, there is a 50% reduction in total SOD activity after exposure to curcumin for 24 hours. This response was seen for both 5 and 10 μg/ml curcumin. Moreover, there is no effect of curcumin on the generation of superoxide in this assay (Fig. 2.3B).

Taken together, these studies of the catalase A and SOD enzymes suggests that curcumin is not acting as an anti-oxidant in *D. discoideum* cells.

III. Curcumin negatively regulates antioxidant enzyme mRNA levels.

Based on the results of the catalase A and superoxide dismutase enzyme assays we wanted to determine if this was due to a change in mRNA level. Thus, we measured the mRNA levels of catalase A (catA) and superoxide dismutase (sod) A, B, E and 2 in
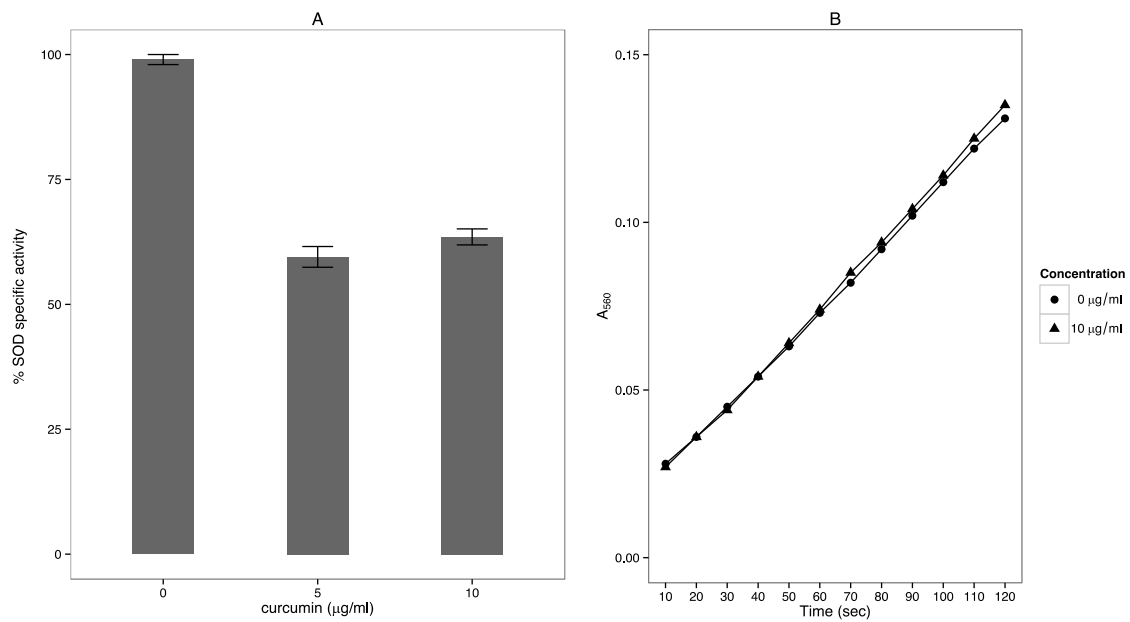
Fig 2.3: Curcumin reduces SOD enzyme activity levels. A) Total SOD enzyme activity in curcumin treated cells (5µg/ml and 10 µg/ml) are reduced by about half relative to untreated cells. B) Curcumin has no effect on the generation superoxide and the rate.

curcumin treated cells. The housekeeping gene Ig7 was used as an internal control. (Teo et al., 2010). The data in Fig. 2.4 show that *cat*A mRNA is reduced by 50% in curcumin treated cells. In similar fashion *sod*A, *sod*B and *sod*2 mRNA were all reduced in response to the curcumin treatment. In contrast, *sod*E mRNA was unchanged in curcumin treated cells. Taken together, these results suggest that curcumin negatively regulates the mRNA levels for 4 antioxidant enzymes, and is consistent with the down-regulation of catalase A and SOD enzymes levels in curcumin treated cells. The data indicate that curcumin is negatively regulating the transcription of these anti-oxidant enzyme genes, and continues to support the idea that curcumin is not acting as an anti-oxidant in these cells**.**

IV. Curcumin upregulates ROS levels.

The data demonstrating that curcumin lowered the levels of antioxidant enzymes (Fig. 2.2 and 2.3) and mRNAs (Fig. 2.4) suggested that this would result in an increase in ROS. It was previously shown that developing *D. discoideum* cells produce superoxide and that this can be measured by the oxidation of XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenl)-2H-Tetrazolium-5-Carboxanilide) (Bloomfield and Pears, 2003). Fig. 2.5A shows that proliferating Ax4 cells also produce superoxide, and that curcumin treatment causes an increase in the level of superoxide produced in a dose dependent fashion. Both 5 and 10 µg/ml curcumin increases the superoxide production showing some increase by as little as 4 hours and much larger increases by 24 hours. Therefore, proliferating cells produce superoxide, and treatment with
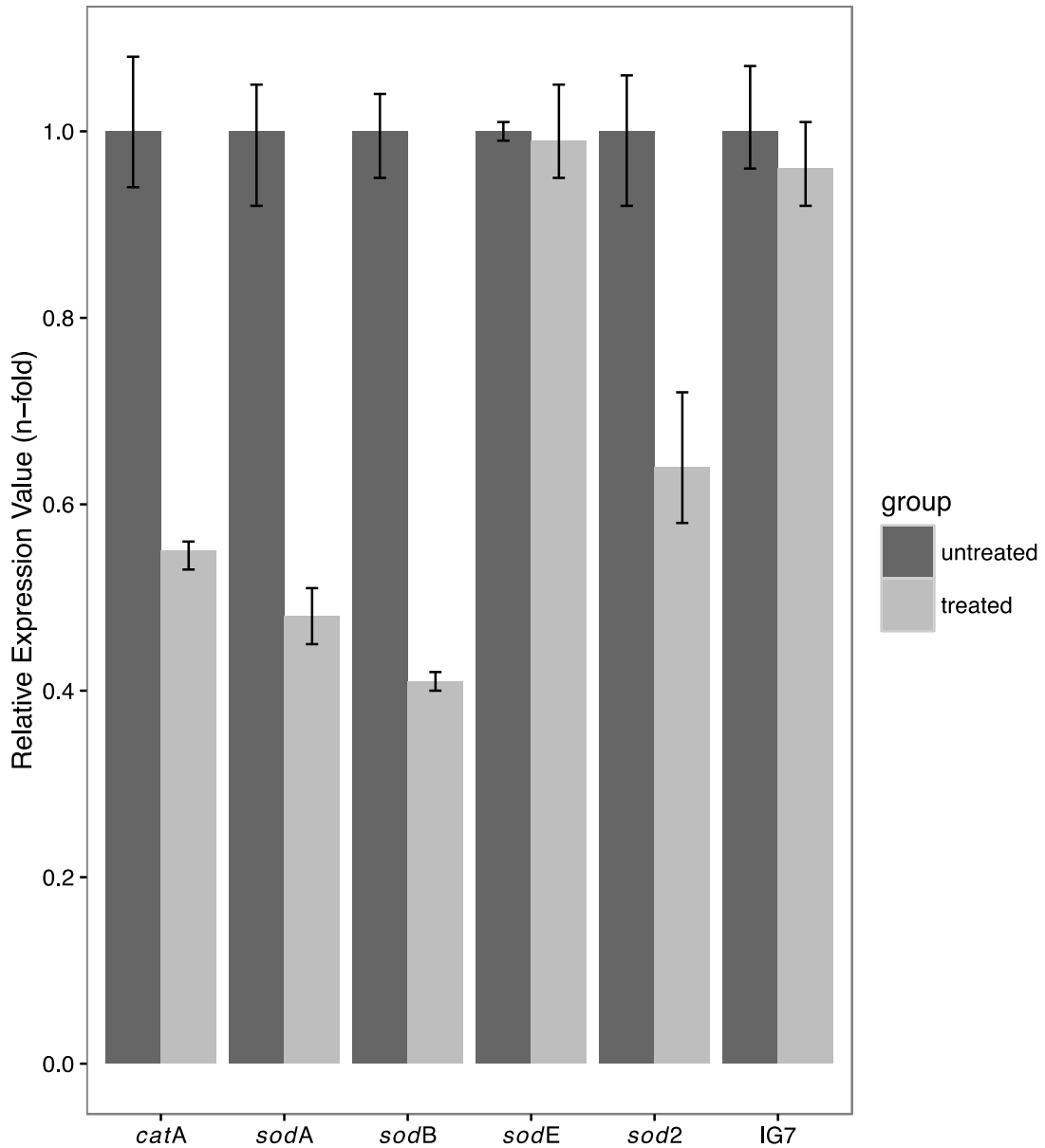
Fig. 2.4: Curcumin negatively regulates antioxidant enzyme RNA levels. Total RNA was prepared from 5×10^6 axenically growing cells treated with 10μg/ml curcumin for 24 hours. The housekeeping gene *Ig7* was used as an internal control. Transcript levels of the antioxidant enzymes, *cat*A, *sod*A, *sod*B and *sod*2 are reduced in cells treated with curcumin. These results indicated that an effect of curcumin on the transcription of the *cat*A, *sod*A, *sod*B and *sod*2 genes.
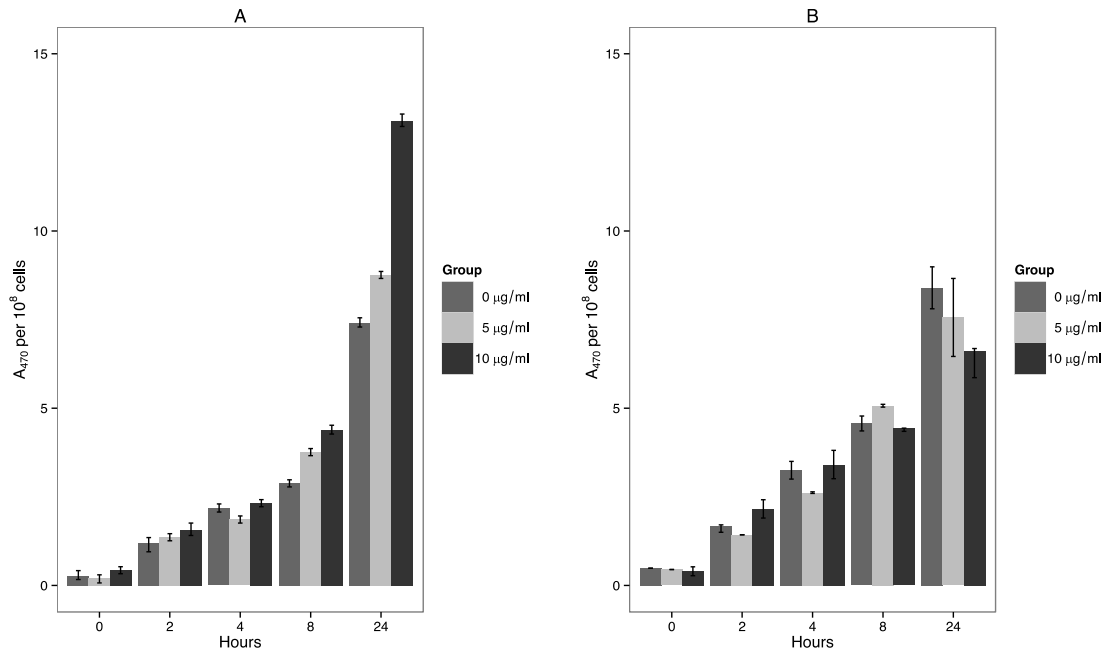
Fig. 2.5: Curcumin upregulates superoxide levels in parental Ax4 cells but not
*pka*C **null cells.** 2×10^6c/ml were treated with curcumin at the indicated

concentrations. The cultures were incubated with XTT for the indicated times and

superoxide generation was measured using an XTT assay. The results shown here

indicate that curcumin increases the level of ROS (superoxide) in a dose-dependent

manner. Both cell types were analyzed in parallel. A) Ax4 cells B) *pka*C null cells.

curcumin results in increased production of superoxide with kinetics that resemble the kinetics of the decrease in the superoxide dismutase enzyme activity and mRNAs. Fig. 2.5B is discussed in section **XI**. below.

The effect of curcumin on the level of H_2O_2 was also determined. This is the first reported determination of H_2O_2 in *D. discoideum*. Figure 2.6A shows that there is a robust increase in H_2O_2 in cells treated with time, and there is a clear dose dependent effect of curcumin seen at 24 hours. The pattern of H_2O_2 accumulation in curcumin treated cells is complementary to the pattern of decrease in the level of catalase in the presence of curcumin. Thus, both H_2O_2 and superoxide increase in curcumin treated cells as predicted by the decrease in the levels of the enzymes that degrade these reactive oxygen species, which strongly supports the notion that curcumin is not acting as an anti-oxidant. Fig. 2.6B is discussed in section **XI**. below.

V. Oxidants have a different effect on proliferating cells than curcumin.

The data from enzyme and mRNA measurements indicted that curcumin was not acting as an anti-oxidant in *D. discoideum* cells. There are previous suggestions that curcumin can act as a pro-oxidant in some situations (Aggeli et al., 2013; Khan et al., 2012; Yoshino et al., 2004). To ask whether the increase in superoxide and H_2O_2 production seen following treatment of cells with curcumin was the factor regulating the decrease in antioxidant enzyme activities and mRNAs (Figs. 2.2. 2.3, and 2.4), proliferating cells were treated with two pro-oxidants, ethidium bromide and menadione. Treatment with both pro-oxidants resulted in an increase in catalase
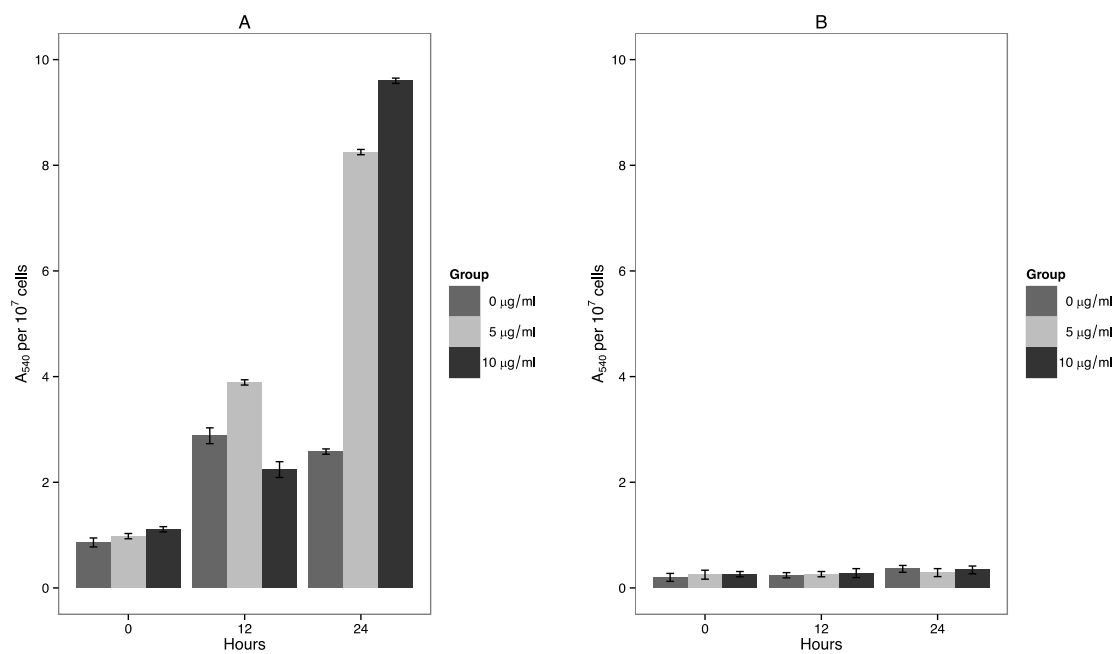
Fig. 2.6: Curcumin upregulates H_2O_2 levels in parental Ax4 cells but not *pka*C null cells. Both cell types were analyzed in parallel. A) Ax4 cells B) *pka*C null cells

specific activity (Fig. 2.7A and B). This is in contrast to the previously observed decrease in catalase enzyme specific activity in the presence of curcumin. Like mammalian cells, *D. discoideum* appears to respond to oxidative stress by upregulating antioxidant enzymes (Mates et al., 1999). Figure 2.7 C and D show that both ethidium bromide and menadione have an inhibitory effect on cell proliferation. Clearly inhibition of proliferation is not the cause of the decrease in the antioxidant enzymes in curcumin treated cells. This result indicates that the increased generation of ROS by curcumin is not the cause of the reduction in the antioxidant enzyme RNAs and enzymes. Rather it suggests that the increase in ROS is due to the reduction of antioxidant enzymes.

VI. The anti-oxidant N-acetylcystiene affects cells differently than curcumin and does not reverse the effects of curcumin.

Based on the observation that curcumin reduced the catalase A and SOD levels, and increased the superoxide production in cells, it was of interest to see if these physiological responses could be reversed with the antioxidant N-acetylcystiene (NAC). NAC at 326 µg/ml (2 mM) has been shown to inhibit production of ROS in human SH_SY5Y neuroblastoma cells induced by chemicals (Murata et al., 2015). NAC treatment (between 100 µg/ml and 400 µg/ml) had no effect on cell proliferation in *D. discoideum* (Fig. 2.8 A and B), indicating that ROS can be inhibited for up to 4 days without a significant effect on cell proliferation. Addition of NAC did not reverse the reduction of catalase A specific activity by curcumin at any of the doses tested (Fig. 2.8 C). Previous studies from this laboratory showed that NAC could inhibit the terminal stages of multicellular development, but required concentrations 15 fold
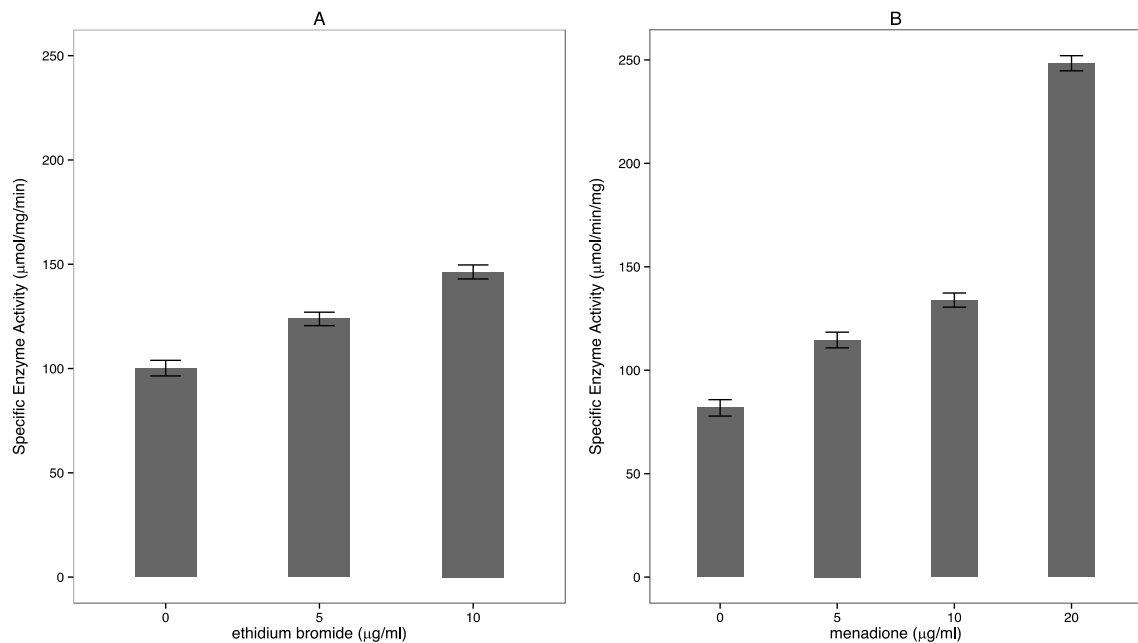
Fig. 2.7: Oxidants have a different effect on cells than curcumin A) Catalase A activity is increased in cells treated with ethidium bromide for 24 hours in a dose dependent manner. B) The same effect is observed with treatment with another oxidant, menadione. These results indicate that *D. discoideum* cells, like mammalian cells, respond to oxidative stress by upregulating antioxidant enzymes (Mates et al., 1999) and that the effect curcumin has on catalase A enzyme specific activity (Fig 2.2) does not appear to be the direct result of oxidative stress.
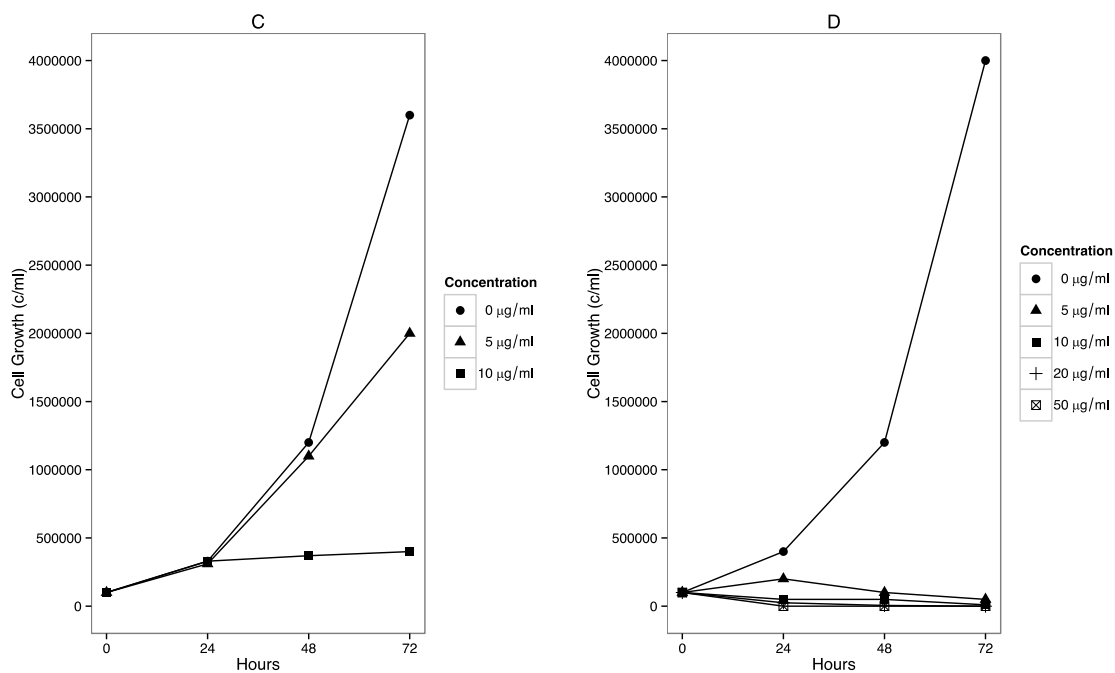
Fig. 2.7 cont'd: Cell proliferation is inhibited in parent Ax4 cells treated with pro-oxidants. C) Ethidium bromide at 5 and 10 μg/ml inhibits cell proliferation of parent cells. D) Menadione at 5, 10, 20 and 50 μg/ml inhibits proliferation of parental cells.
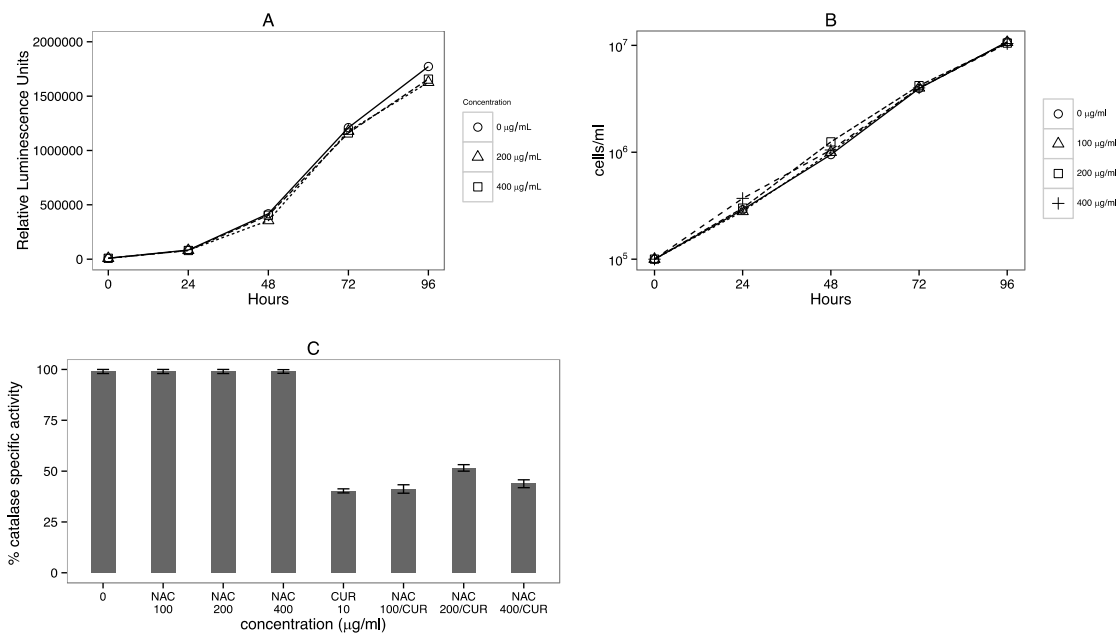
Fig. 2.8: The antioxidant NAC affects cells differently than curcumin and do not reverse the oxidant effect of curcumin. The antioxidant, N-acetylcysteine, known to counter the effect of oxidative stress does not have any effect A) viability or B) proliferation of *D. discoideum* cells. C) NAC did not counter the effect of curcumin of cells treated with curcumin for 24 hours indicating that the effect of curcumin on catalase A specific enzyme activity was not directly due to oxidative stress.

higher for this effect (Garcia et al., 2003). Thus, it was possible that the concentrations

of NAC used in Fig. 2.8 A and B were not high enough to elicit an antioxidant response.

NAC concentrations were increased up to 7000 μg/ml. Indeed, at these higher

concentrations, there is inhibition of cell proliferation (Fig. 2.8 D). However, even at

these concentrations NAC does not reverse the effect of curcumin on the level of

catalase A enzyme activity (Fig. 2.8 E). At the highest concentrations NAC is lethal.

These data and those in Fig 2.7 strongly suggest that curcumin is regulating the level

of antioxidant enzymes through a mechanism independent of ROS.

VII. The superoxide scavenger, XTT, does not reverse the inhibitory effect of curcumin on cell proliferation.

In a previous study, XTT was shown to scavenge superoxide in *D. discoideum* cells

(Bloomfield and Pears, 2003). To investigate whether the increased accumulation of

superoxide was the cause of the inhibitory effects of curcumin, cells were grown in

the presence of XTT. Fig 2.9 shows that XTT is not able to reverse the inhibitory effect

of curcumin suggesting that curcumin's inhibitory effect is not as a result of increased

accumulation of superoxide.

VIII. The study of candidate genes reveals that PKA is involved in the cellular response to curcumin.

There are a number of paths to investigate the underlying mechanisms regulating

curcumin sensitivity that take advantage of the genetic tractability in *D. discoideum*.

These include 1) a candidate gene approach using the now extensive library of
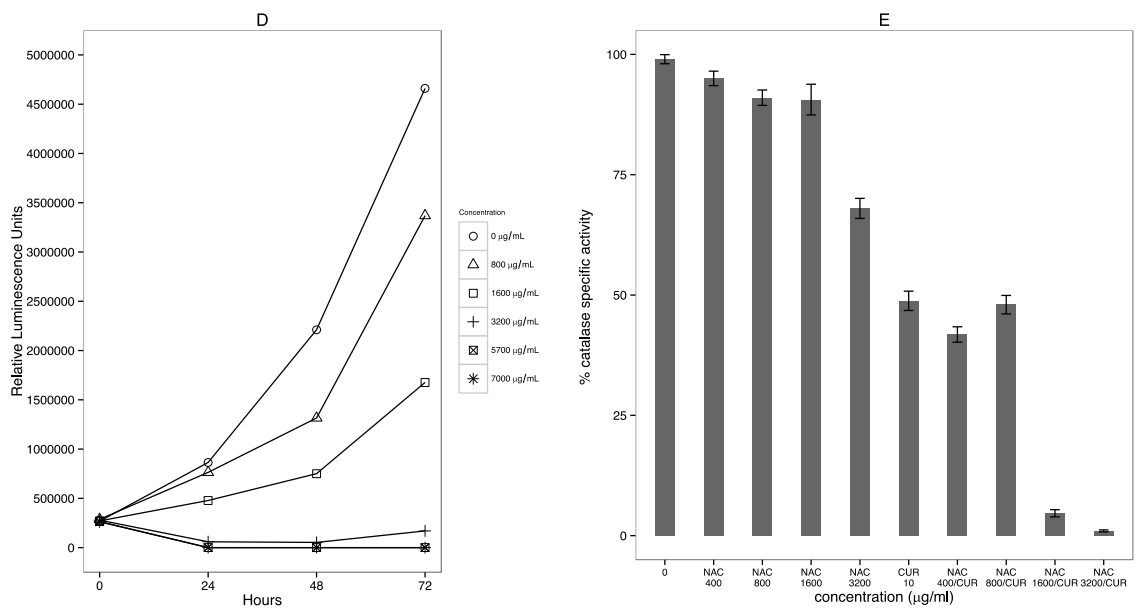
Fig. 2.8 cont'd: NAC at higher concentrations does not reverse the oxidant effect of curcumin. D) Increased NAC concentrations inhibit cell proliferation. E) However, these increasing concentrations of NAC still do not counter the effect of cells treated with curcumin for 24 hours confirming that the effect of curcumin on catalase A specific enzyme activity was not directly due to oxidative stress.
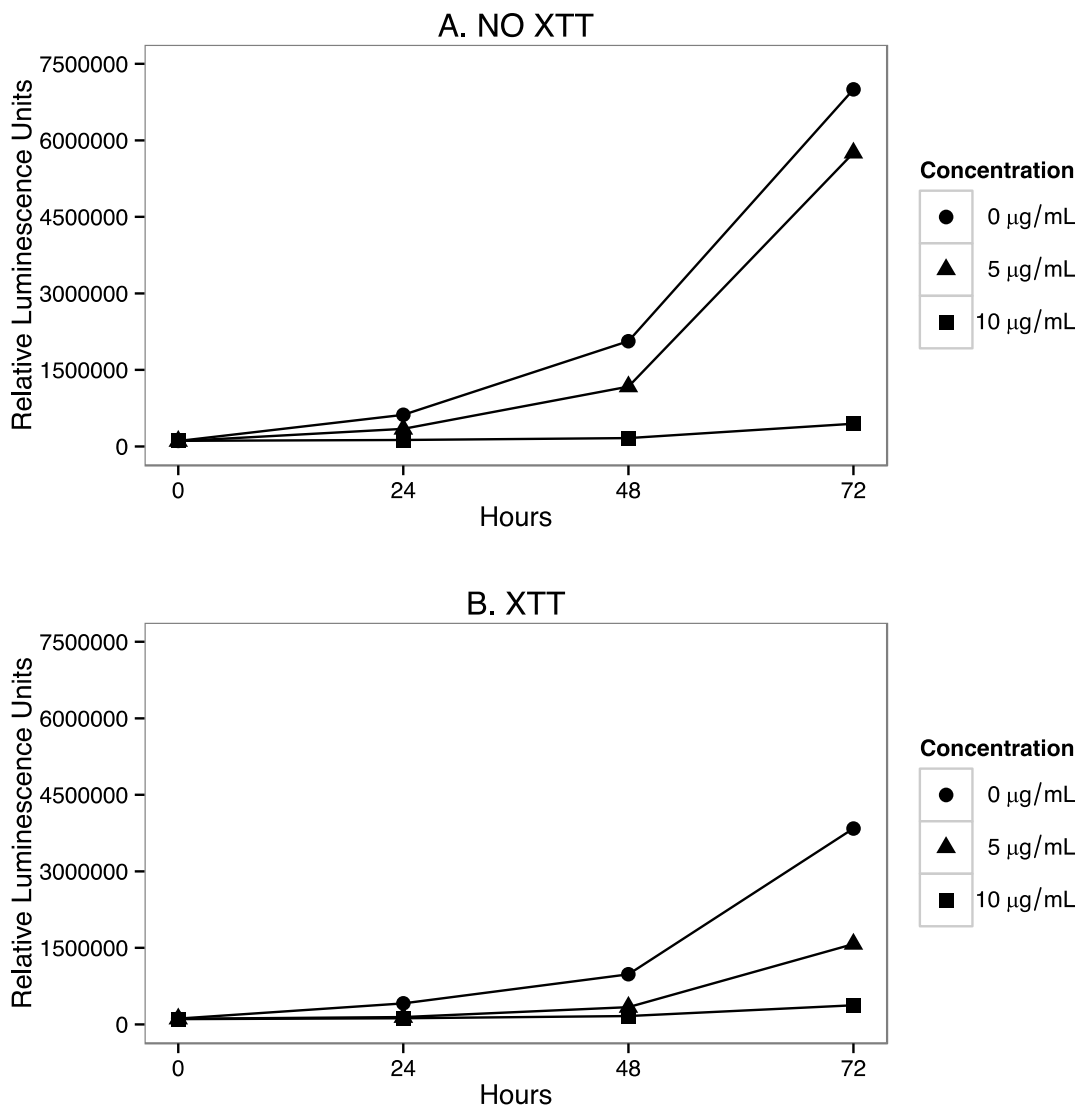
Fig. 2.9: XTT does not reverse the inhibitory effect of curcumin. To assess whether superoxide accumulation was the factor regulating the inhibitory effect of curcumin, parental strain Ax4 was tested for growth in the presence of curcumin and XTT. XTT was not able to reverse curcumin's inhibitory effect.

isogenic mutant strains that each differ from the parent by one gene (see Dictybase.org); 2) using insertional mutagenesis (REMI; Kuspa and Loomis, 1992) to randomly inactivate genes in cells which can subsequently be identified by virtue of the cells' increased resistance to curcumin; and 3) employ a gene expression strategy such as microarrays or RNAseq (Parikh et al., 2010) to identify alteration of the expression patterns of genes and gene networks in response to curcumin. All of these approaches have been successfully employed in *D. discoideum* to investigate problems in cell and developmental biology including studies of importance to human diseases such as cancer and chemotherapy (Alexander and Alexander, 2011).

A variety of existing mutant strains with deleted or over-expressed genes in known regulatory/signaling pathways or genes involved in generating or scavenging ROS were tested for their response to curcumin. The results are shown in Fig. 2.10.

Parent strain Ax4. Fig. 2.10 confirms what was previously presented in Fig. 2.1. The parental Ax4 cells showed a dose dependent inhibition of cell proliferation by curcumin so that by 10 µg/ml there was significantly reduced cell proliferation at 72 hours. The same results were obtained with Ax2, another strain of parental cells.

*erk*B null mutant. The *erk*B gene is a key regulator of development (Segall et al., 1995). *erk*B activates adenylyl cyclase as well as inhibiting the regA phosphodiesterase (Loomis, 1998). This mutant also showed a dose dependent inhibition of cell proliferation similar to the parent cells.

66

*reg*A null mutant. RegA is a phosphodiesterase encoded by *reg*A (Shaulsky et al., 1996). RegA negatively regulates the activity of protein kinase A (PKA) by catalyzing the hydrolysis of the PKA activator cAMP (Shaulsky et al., 1998). Interestingly, a random insertional mutagenesis study done in *D. discoideum* to investigate the molecular mechanisms underlying resistance to the anti-cancer drug cisplatin, identified *reg*A as one of the genes involved in the cellular response to cisplatin (Li et al., 2000). However, *reg*A mutants show a similar dose dependent effect of curcumin on cell proliferation as seen with the parent cells.

*reg*A null mutant*/erk*B null mutant. The double null mutant strain generated from an *erk*B null parental strain shows a modest increase in cell proliferation at both 5 and 10 μg/ml curcumin.

*yak*A null cells. *yak*A encodes a protein kinase which is required for the growth to development transition in *D. discoideum* (Souza et al., 1998) and has been implicated in the modulation of stress response in *D. discoideum* (Taminato et al., 2002). In that study, *yak*A mutants were shown to have increased sensitivity to nitrosoative/oxidative stress relative to the parent strain. This suggested that there might be a relationship of *yak*A to curcumin sensitivity based on the increase in ROS in curcumin treated cells (Fig. 2.5 and 2.6). However, *yak*A null mutants show similar sensitivity to curcumin as the parent cells.

Sphingosine kinase A over-expressor. A number of studies have reported that bioactive sphingolipids are involved in the cellular response to curcumin in a variety of mammalian cell types (Moussavi et al., 2006; Shakor et al., 2014; Yang et al., 2012). Sphingolipids have been studied in *D. discoideum*, and the sphingosine kinase A over-expressing strain was shown to have increased resistance to the chemotherapeutic drug cisplatin (Min et al., 2005a). These cells showed an increase in resistance to curcumin at both 5 and 10 μg/ml.

*cat*A null mutant. Strain IR41 lacks the *cat*A gene which is expressed uniformly in mitotically dividing cells and throughout development (Garcia et al., 2002). It has been studied extensively by this laboratory in terms of its role in resistance to methanol and unrelated compounds such as thiabendazole, and its role in resistance to hydrogen peroxide and other cellular stresses (Garcia et al., 2003; Garcia et al., 2000). Based on the finding in Figs. 2.2, it was possible that cells lacking *cat*A would have an altered response to curcumin. However, the resistance of the *cat*A null cells to curcumin is identical to that of the parent strain. Thus, *cat*A does not appear to be a primary defense against the effects of curcumin.

sodA overexpressor. A strain overexpressing the superoxide dismutase A gene was described in an earlier study (Bloomfield and Pears, 2003). *sod*A is one of seven superoxide dismutase genes in *D. discoideum*. The *sod*A overexpressing strain was characterized and shown by western analysis to produce an increase in SODA protein compared to the parent strain. *sod*A was one of the genes shown in Fig. 2.4 to be

down-regulated after exposure to curcumin, and we have also documented in Fig. 2.5 that superoxide (SO) is up-regulated in response to curcumin in a temporally correlated way to the loss of SOD. Examination of this mutant showed an increase in resistance to curcumin where the cells grew well in the presence of 5 and 10 µg/ml curcumin in contrast to the parent cells.

PKA (*pka*R null, *pka*R over-expressor and *pka*C null mutant). The cAMP-dependent protein kinase A is known to regulate the activity of a wide number of proteins. This well characterized enzyme in *D. discoideum* plays a central role in development affecting aggregation, chemotaxis, and spore differentiation (Loomis, 1998; Mann et al., 1997), and has important regulatory functions in human, zebrafish, Drosophila, and yeast. PKA is made up of a single regulatory subunit (PKA-R) and a single catalytic subunit PKA-C) (Harwood et al., 1992; Mutzel et al., 1987). cAMP binding to PKA-R causes the dissociation of PKA-C rendering PKA-C active. A mutant strain, *pka*RΔ, harboring a null mutation in the regulatory subunit showed similar sensitivity to curcumin as the parent. However, two strains, one carrying a null mutation in *pka*C and another over-expressing the regulatory subunit *pka*R both showed greatly increased resistance to curcumin at 10 µg/ml compared to the parent strain.
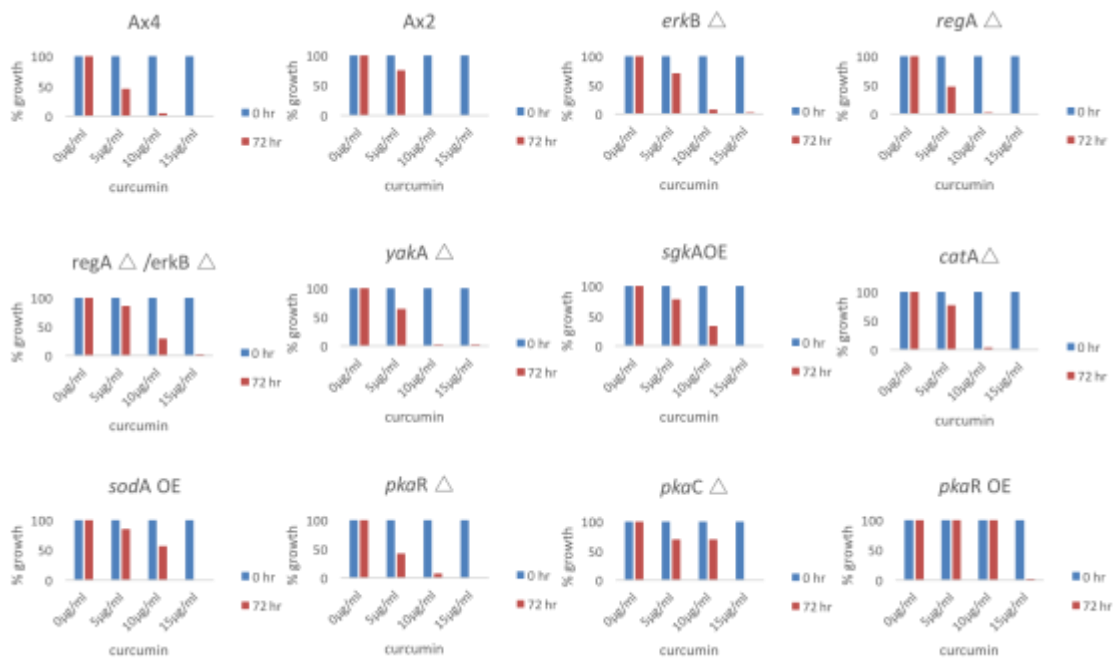
Fig. 2.10: Examination of candidate genes reveals that PKA is required for the cellular response to curcumin. The indicated mutants are described in detail in the text, were tested for their sensitivity to curcumin over time. Each mutant was tested multiple times and in parallel with the wild-type Ax4 strain. Cell proliferation was measured using the CellTiter-Glo® assay described in Materials and Methods (Min et al., 2006). Δ = null mutant, OE = over-expressing mutant.

IX. Curcumin resistance is mediated by inactivation of PKA.

The increased resistance of the *pkaC* null and *pkaR OE* mutants to curcumin in liquid medium suggested that curcumin resistance was mediated by PKA (Fig. 2.10). To validate this, we performed a different viability assay by clonally plating these mutant strains in association with *Klebsiella aerogenes* in the presence of curcumin. Results from this experiment confirmed that cells with an inactive form of PKA (*pka*C null or *pka*R OE) showed increased resistance to curcumin than the parent strain (Fig. 2.11).

Many studies to investigate PKA function have employed the use of the PKA inhibitor, KT 5720 to block PKA activity (Ahn et al., 2016; Deming et al., 2008; Dodge-Kafka et al., 2005). To mimic the curcumin resistant phenotype of *pka*C null cells pharmacologically, we treated parental cells with both curcumin and KT 5720 (Sigma, St. Louis MO) and measured cell viability. KT 5720 has been reported to inhibit PKA with an inhibition constant of 60nM (Kase et al., 1987). Fig. 2.12 A shows that treatment of parental cells with concentrations of KT 5720 up to 640 nM have no effect on cell proliferation. In addition, the same concentrations of KT 5720 were not able to increase the resistance of cells to curcumin. (Fig. 2.12 B). However, it is not known if KT 5720 inhibits *D. discoideum* PKA.
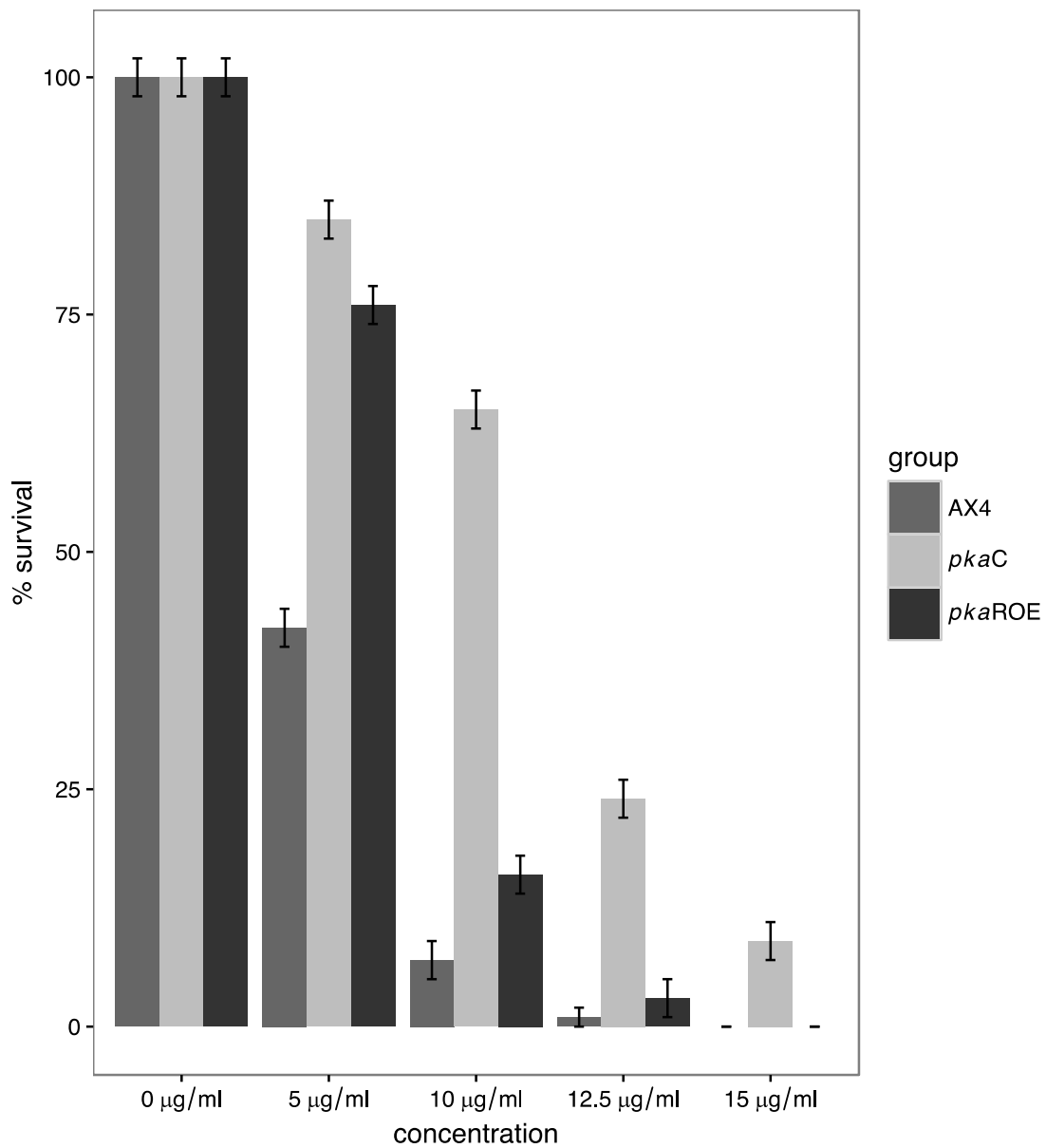
Fig. 2.11: Validation that the *pka*C null and *pkaR*-OE mutants are more resistant to curcumin. *pka*C null and *pkaR*-OE are more resistant to curcumin when clonally plated in association with *Klebsiella aerogenes* on SM plates containing curcumin at the indicated concentrations.
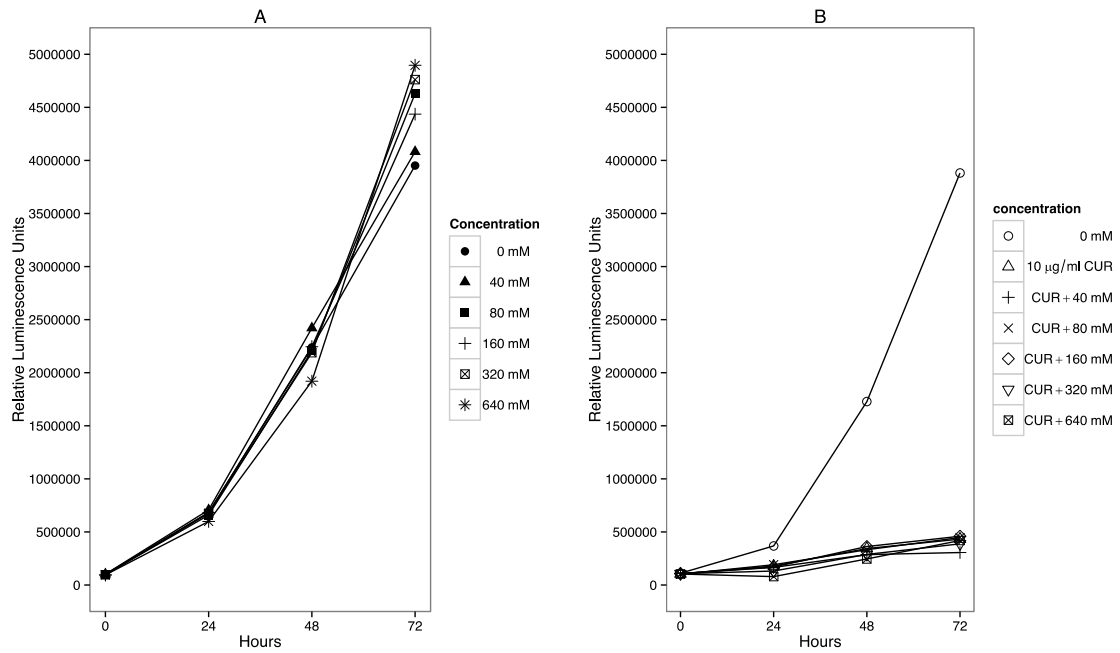
Fig. 2.12: PKA inhibitor KT 5720 does not mimic curcumin resistant phenotype of *pkaC* null cells. A. The PKA inhibitor KT 5720 does not inhibit cell proliferation of parental cells. **B**. KT 5720 does not increase resistance to curcumin.

X. The catalase A and SOD enzyme activities are not affected by curcumin in *pka*C⁻ null cells

*pka*C⁻ null cells treated with curcumin were assayed for the level of catalase A enzyme activity. The data demonstrates that concentrations up to 10 μg/ml of curcumin did not affect catalase A enzyme activity in *pka*C⁻ null cells (Fig. 2.13), in contrast to the decrease in *cat*A specific activity in the parental cells, confirming previously seen results where the level of catalase A enzyme activity in Ax4 cells was reduced (Fig. 2.2).

*pka*C null cells treated with curcumin for 24 hours showed only a 10% decrease in total SOD enzyme activity compared to the parental cells treated and assayed in parallel (Fig. 2.14). Although this assay is measuring all the SOD enzymes expressed in the cell, the data support the idea that the SOD enzymes, like the catalase A enzyme, are not all down regulated by curcumin in the *pka*C null cells.

XI. ROS levels in *pka*C⁻ null cells are not upregulated by curcumin

Previous data had shown that catalase A and SOD enzyme specific activities in Ax4 cells decrease upon treatment with curcumin (Fig. 2.2 and 2.3), resulting in an increase in SO and H_2O_2 levels (Figs. 2.5A and 2.6A). Curcumin had little to no effect on catalase A and SOD activity in *pka*C⁻ null cells (Fig. 2.13 and 2.14), suggesting that ROS may not be up-regulated as observed with parent Ax4 strain. Thus, we tested the *pka*C⁻ null cells for the effect of curcumin on SO production. As shown in Fig. 2.5B
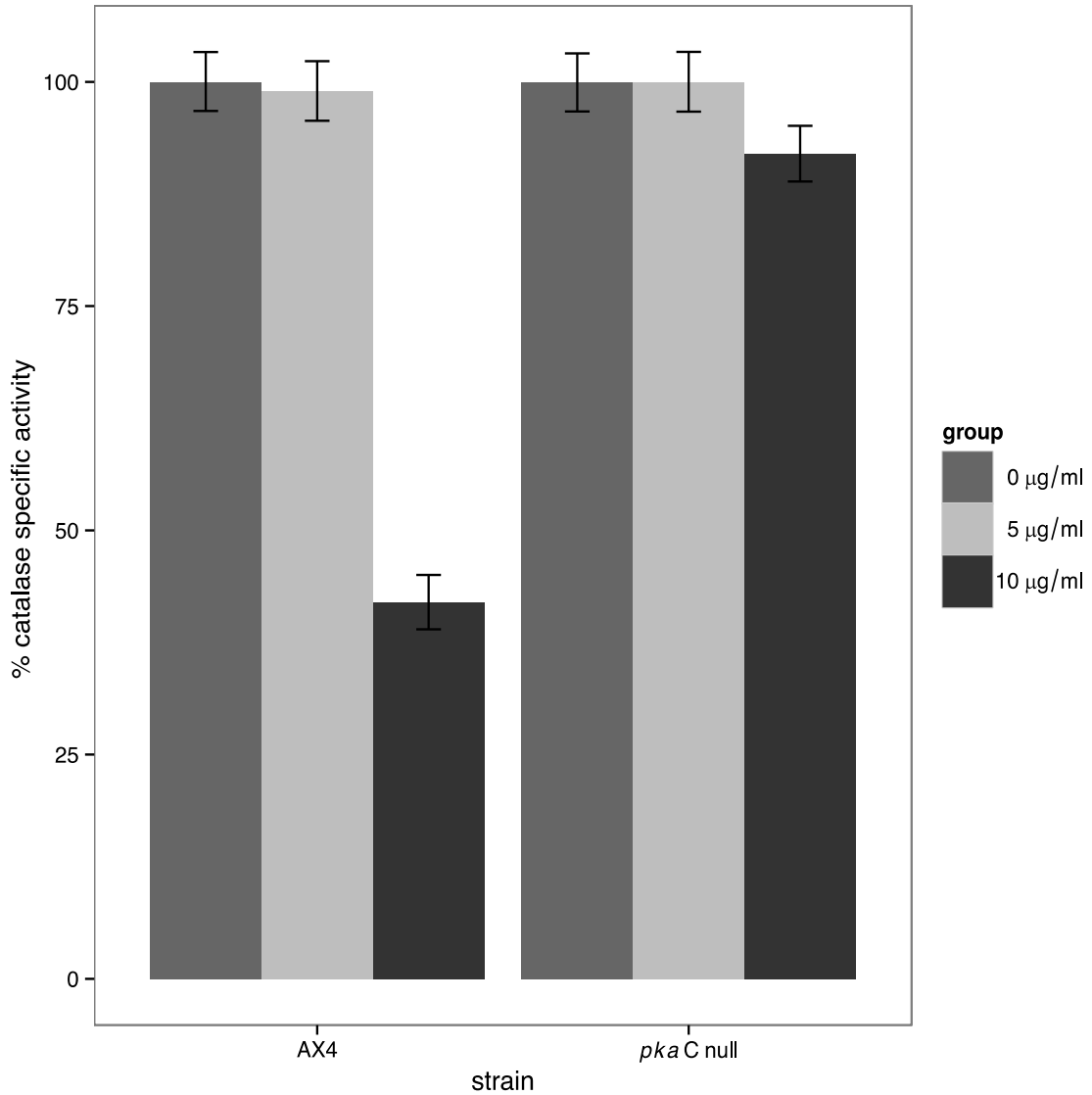
Fig. 2.13:   Catalase A enzyme levels are unchanged in curcumin treated *pka*C null cells. Unlike WT cells, *pka*C null cells treated with curcumin for 24 hours did not show a change in catalase A specific enzyme activity.
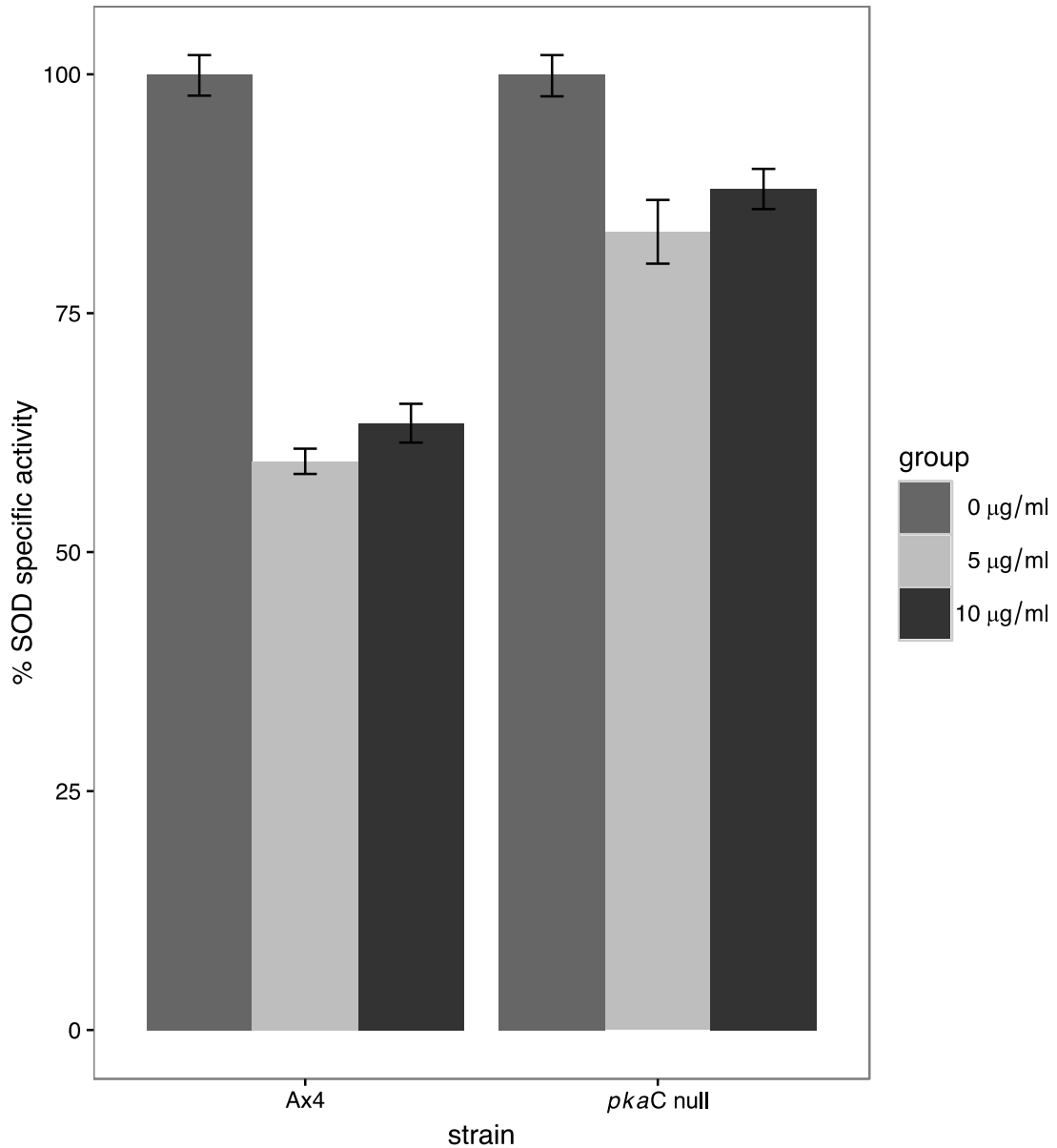
Fig. 2.14: **SOD enzyme levels in *pka*C null cells treated with curcumin.** Unlike WT cells that exhibit a 50% reduction in total SOD enzyme activity, *pka*C null cells treated with curcumin for 24 hours only exhibited a 10% reduction in total SOD activity.

curcumin does not increase the level of SO being generated in the *pkaC* null cells compared to the parental Ax4 cells (Fig. 2.5A). These results are in agreement with the relative lack of SOD enzyme down-regulation observed in curcumin treated *pka*C null cells (Fig. 2.14). In similar fashion, as seen in Fig. 2.6B the level of H_2O_2 does not increase in the curcumin treated *pka*C null cells compared to the parental Ax4 cells (Fig. 2.6A). Indeed, this mutant shows virtually no increase in H_2O_2 level in the presence or absence of curcumin. Thus, *pka*C null cells do not respond to curcumin by up-regulating the level of ROS, presumably as a result of not down-regulating their catalase A and SOD enzyme levels.

XII. Isolation of curcumin resistant mutants.

D. discoideum offers a tractable genetic system for the interrogation of the mechanism of action of drugs and botanicals such as curcumin. Because curcumin inhibits cell proliferation it was of interest to see if mutants could be isolated that would grow in the presence of curcumin at a concentration that inhibits the proliferation of the parental Ax4 cells. Curcumin was titrated for its effect on cell growth when plated on SM agar plates where the cells are grown in association with a bacterial food source. It was determined that at a concentration of 15 μg/ml curcumin added to SM agar plates only 5 in 10^6 cells survives and grows (Fig. 2.15 A). Several of these strains were isolated and tested by clonal plating and shown to have plating efficiencies of 20-30%, whereas the parental Ax4 cells exhibited plating efficiency of 0% (Fig. 2.15 B). These results suggest that the surviving cells were spontaneous mutants. This
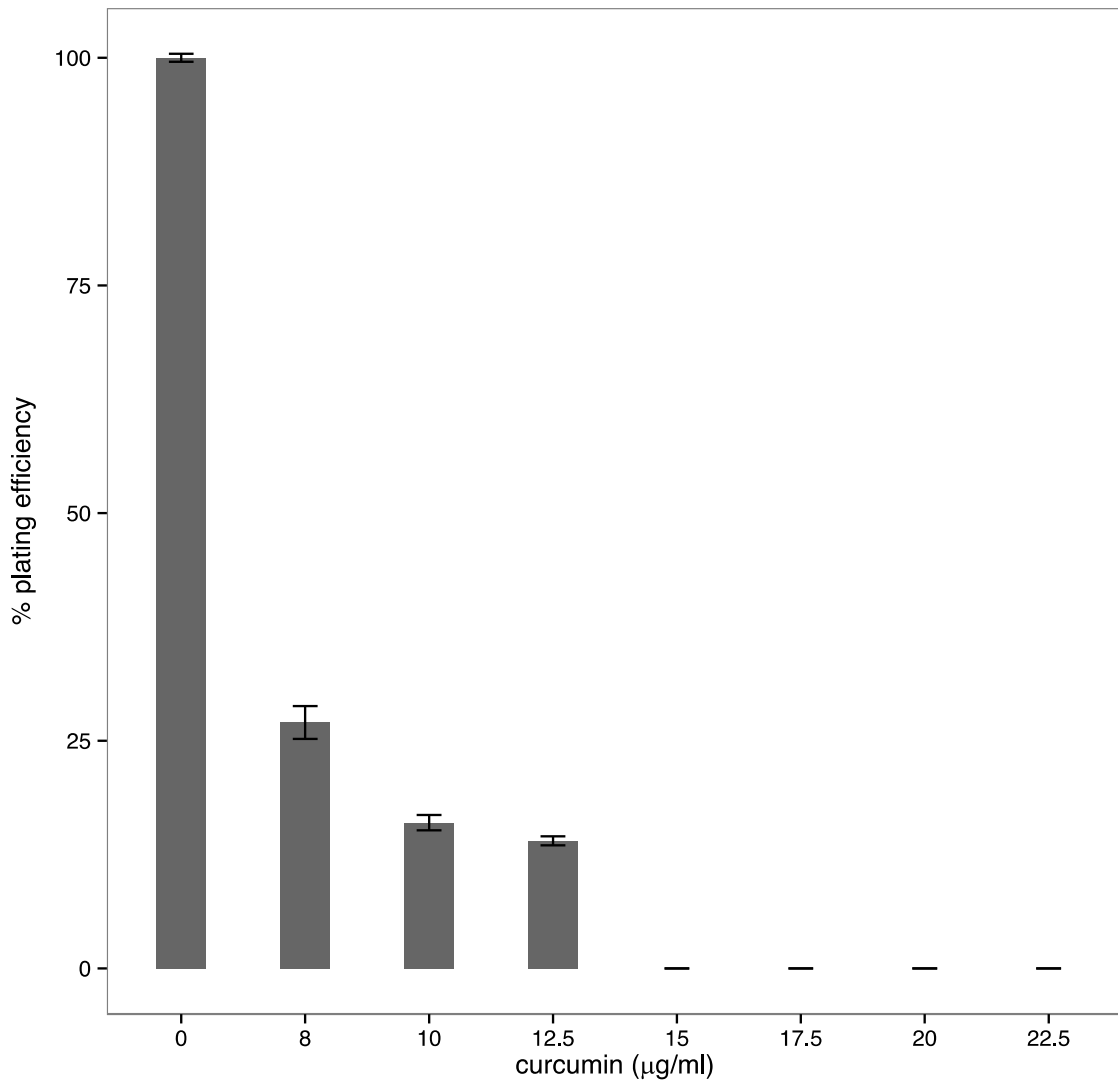
Fig. 2.15 A: Curcumin resistant mutants can be isolated. To assess whether spontaneous curcumin resistant mutants could be isolated, 10^2 cells were plated in association with *K. aerogenes* on SM plates containing curcumin at the indicated concentrations. This figure shows plating efficiency on curcumin as a percentage of the number growing on SM agar plates without curcumin.
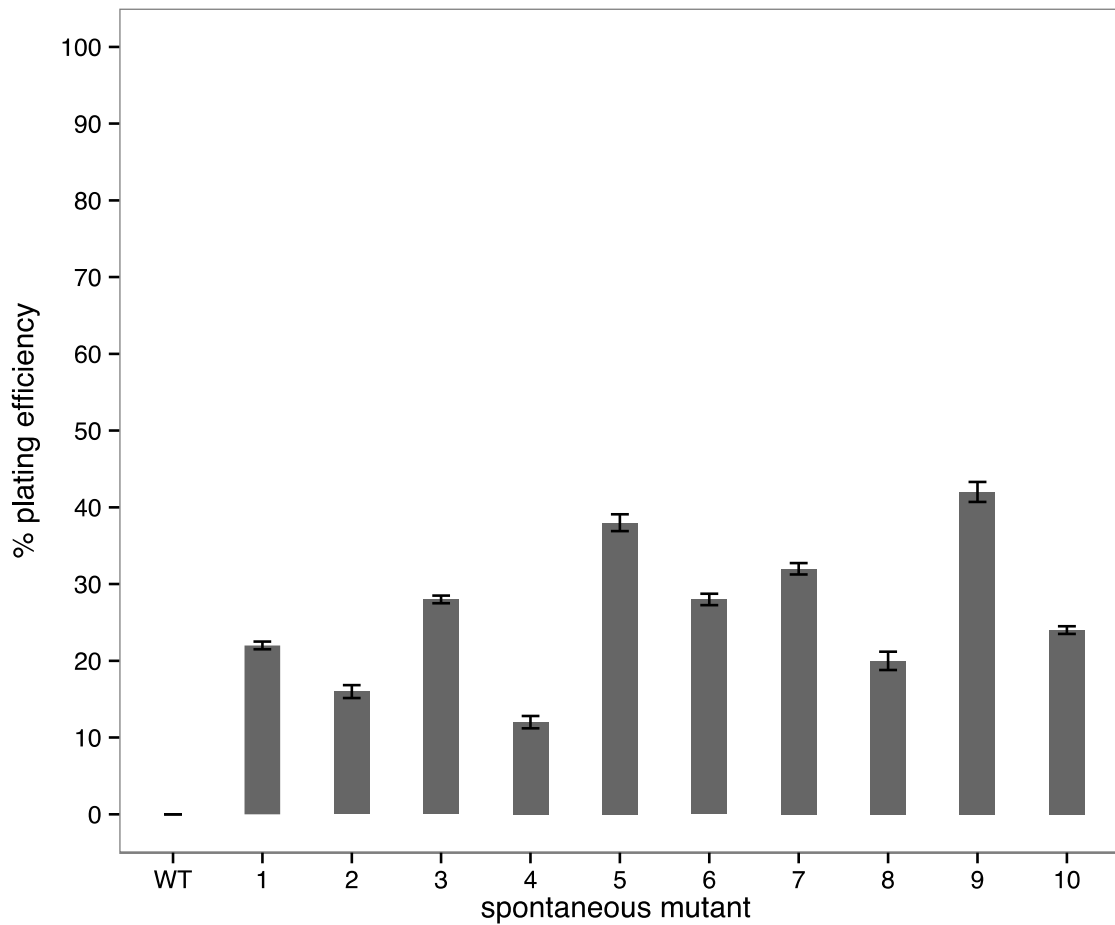
Fig. 2.15 B: Retest of the effect of curcumin on spontaneous curcumin resistant mutants. Spontaneous curcumin resistant mutants were retested for growth on SM agar plates containing 15 μg/ml curcumin and continued to show resistance to curcumin at plating efficiencies mostly between 20 and 30%.

level of penetrance is typical for mutants exposed to exogenous compounds that inhibit growth including methanol resistant mutants, which has been extensively characterized (Garcia et al., 2000; Garcia et al., 2002).

XIII. Restriction Enzyme Mediated Integration (REMI)

Electroporated cells were subjected to blasticidin selection and then spread onto SM agar plates containing 15 µg/ml curcumin in association with *K. aerogenes*. Surviving cells were allowed to give rise to single plaques and a total of 58 clones were generated from all four transformations. Subsequently, a total of 12 clones were picked, serially diluted and retested for curcumin resistance on curcumin containing SM agar plates (Fig. 2.16). Two of these clones that exhibited continued curcumin resistance (SM 10 and SM 61) were chosen to identify the site of gene disruption. The inverse PCR approach has not identified the affected genes, and indicates that direct cloning approaches are needed.

XIV. *D. purpureum* is resistant to curcumin at doses that effect *D. discoideum*.

D. purpureum has been studied less extensively than *D. discoideum*. Its genome has recently been sequenced (Sucgang et al., 2011), and its development and transcriptional phenotype have been studied (Parikh et al., 2010). No studies have been done on this species' sensitivity to chemicals or drugs. Remarkably, *D. purpureum* was resistant to the effects of curcumin up to 60 µg/ml (Fig. 2.17), which is 4 times the amount that completely inhibited the proliferation of *D. discoideum* (Fig.
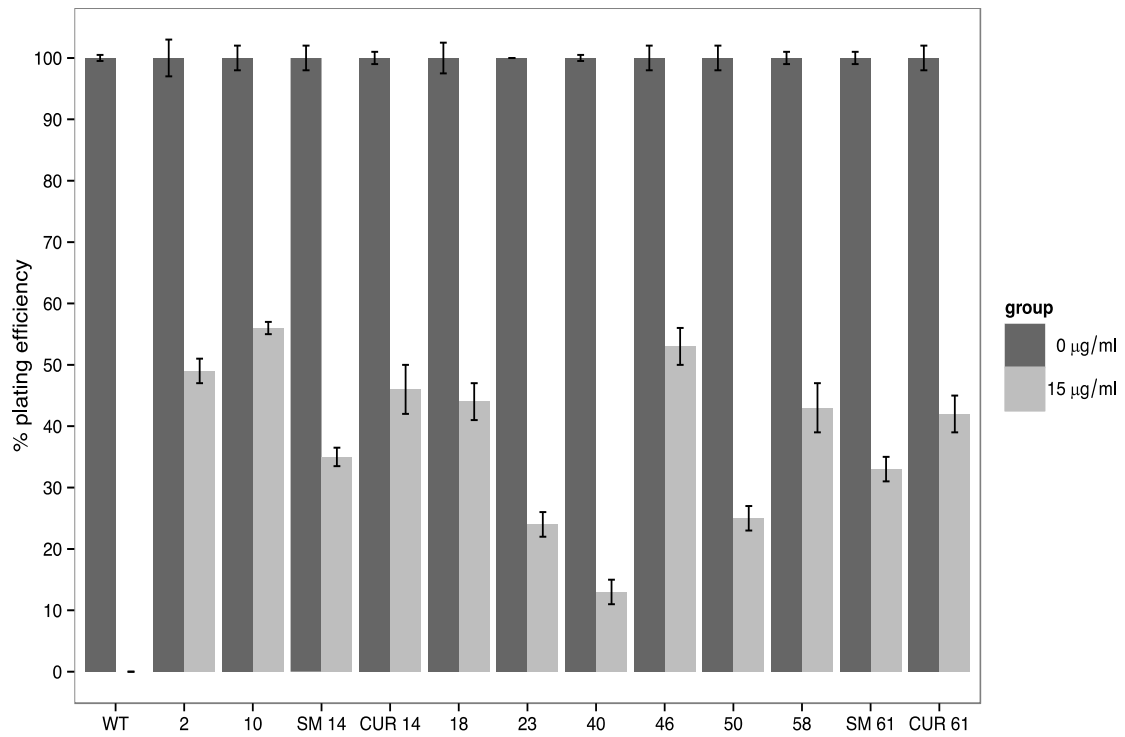
Fig. 2.16: Re-screening of curcumin resistant REMI mutants. Mutants from the REMI mutagenesis that were resistant to curcumin were further tested again for resistance on SM agar plates containing 15 μg/ml curcumin.
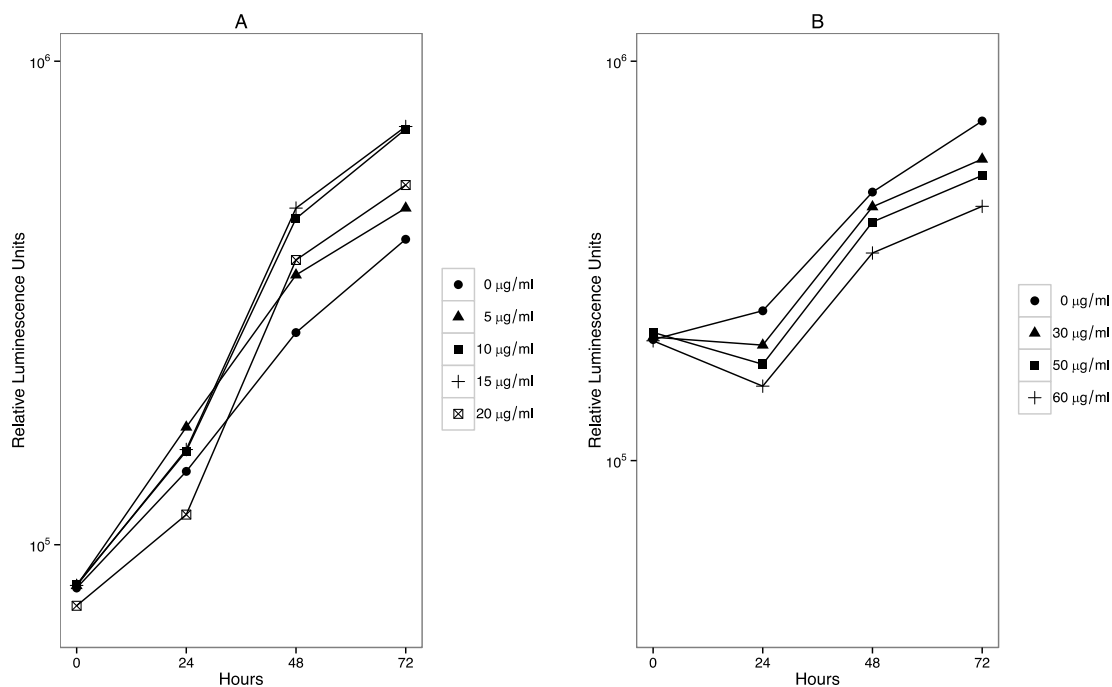
Fig. 2.17: *Dictyostelium purpureum* **is resistant to curcumin**. A) Axenically growing wild-type *D. purpureum* cells were treated with curcumin at the indicated concentrations over three days. Each day cell viability was assayed by measuring ATP in metabolically active cells using Promega's CellTiter-Glo®. B) The experiment was repeated with higher concentrations of curcumin. These results indicate that *D. purpureum* is highly resistant to curcumin in contrast to *D. discoideum*.

2.1). This surprising resistance to curcumin may be useful in unraveling the underlying mechanism of action of this botanical.

XV. RNA Sequencing Analysis

The down regulation of SOD and catalase suggests that curcumin reduces the level of transcription. Thus, we wanted to see what other genes are altered by curcumin at the RNA level and thus to gain more clues of its mechanism of action. To examine time and concentration dependence of *D. discoideum* to curcumin on a genome-wide scale, we performed a RNA-seq analysis. The goal was to identify the time and concentration that show the most effect on transcription, to identify specific genes that are affected, and to correlate these molecular phenotypes with previously discovered cellular physiology.

Triplicate samples were obtained at multiple time points (0, 4, 8 and 12 hour) and multiple concentrations of curcumin (0, 2.5, 5.0, 7.5 and 10.0 μg/ml) (Table 2.5). These parameters were chosen with the aim of providing a broad nuanced view of the effect of curcumin on transcription from times and concentrations that do not have a significant effect on cell proliferation through conditions where the effect on cell proliferation is more profound. As seen in the following data this experimental design worked well.

Biological Replicates	Curcumin concentration (μg/ml)	Time at harvest (hr)	No. of samples
3	0	0, 4, 8, 12	12
3	2.5	4, 8, 12	9
3	5.0	4, 8, 12	9
3	7.5	4, 8, 12	9
3	10	4, 8, 12	9
			TOTAL=48

Table 2.5: Sample preparation for RNA sequencing analysis. Triplicate samples were made for each condition (time and concentration) to be analysed.

XVI. Growth of cells in curcumin and quality of the samples.

Fig. 2.18 shows the growth curves and RNA yields of the cells prepared for the RNAseq experiment. The data are in agreement with previous data (Figs. 2.1 and 2.10) and show that there is a concentration dependent effect of curcumin on cell growth. The sequenced cDNA from the samples was analyzed for consistency between biological replicate samples (Fig. 2.19). In all cases, the biological replicates were excellent with the exception of two samples (one sample of 12 hour and 2.5 µg/ml concentration and one sample of 8 hour and 7.5 µg/ml concentration). Although these samples were not particularly bad we chose to exclude these from the following analyses. When treated samples were compared to the untreated zero-hour samples the differences in transcriptional profile between the treated and control sample is relatively small compared to the massive changes in gene expression seen during the multicellular phase of development (Parikh et al., 2010). This implies that the effect of curcumin, while clearly affecting the transcription of multiple genes, is relatively nuanced.

XVII. Hierarchical Clustering Analysis

The underlying idea when comparing the transcriptional profiles of the samples is to treat each as a molecular phenotype. Each branch of the dendrogram in Fig. 2.20 represents average transcriptional profiles of the replicate samples and the linkage pattern illustrates the relationships between the samples. The data show that there is a clear concentration dependence on transcription with a clear threshold between
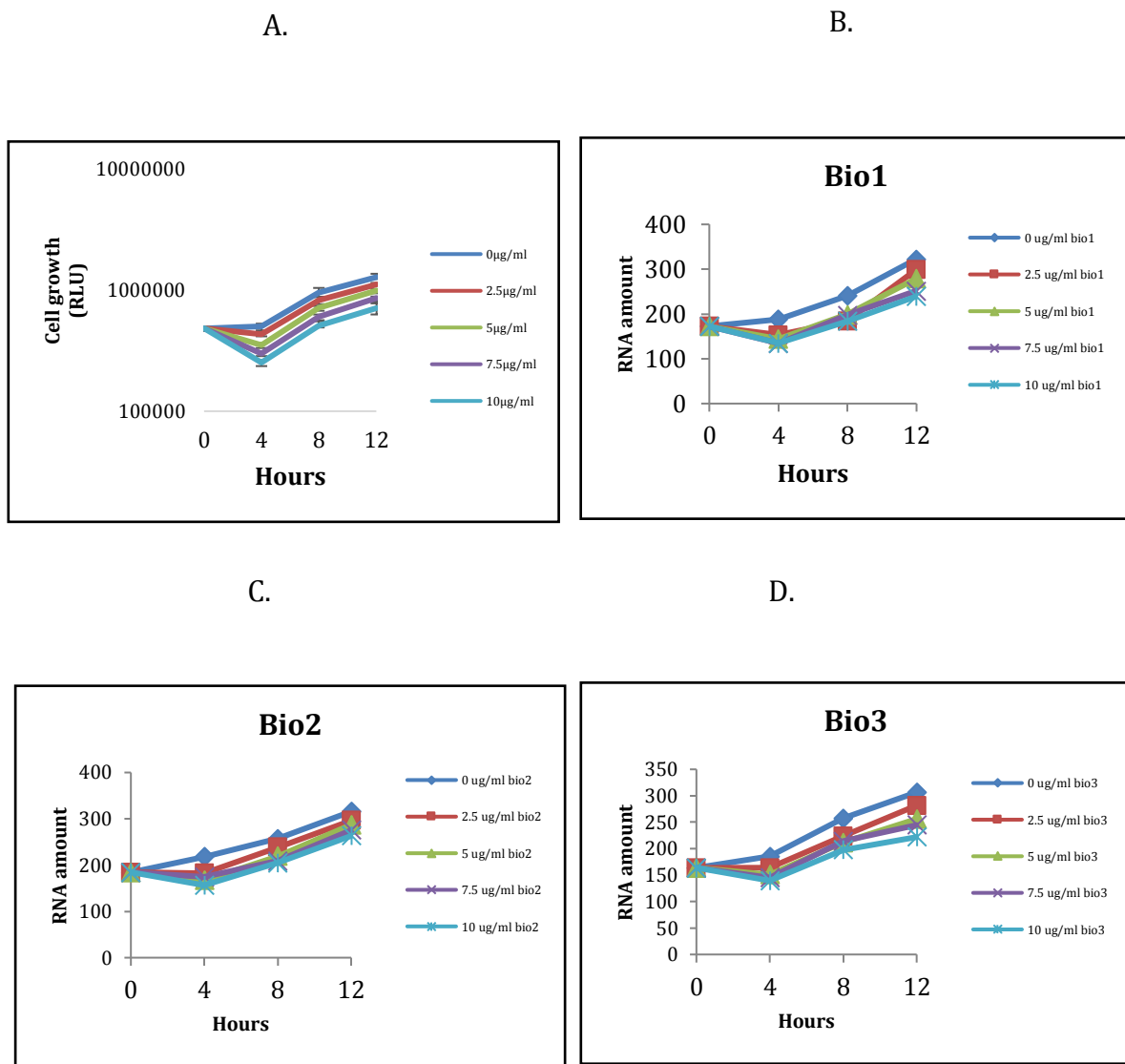
Fig. 2.18: Growth of cells and RNA yields for RNAseq analysis. Samples used for RNA seq analyses were assayed for A) growth and showed similar growth rates to those previously seen in other experiments. RNA yields from triplicate samples B-D) of each condition were similar.
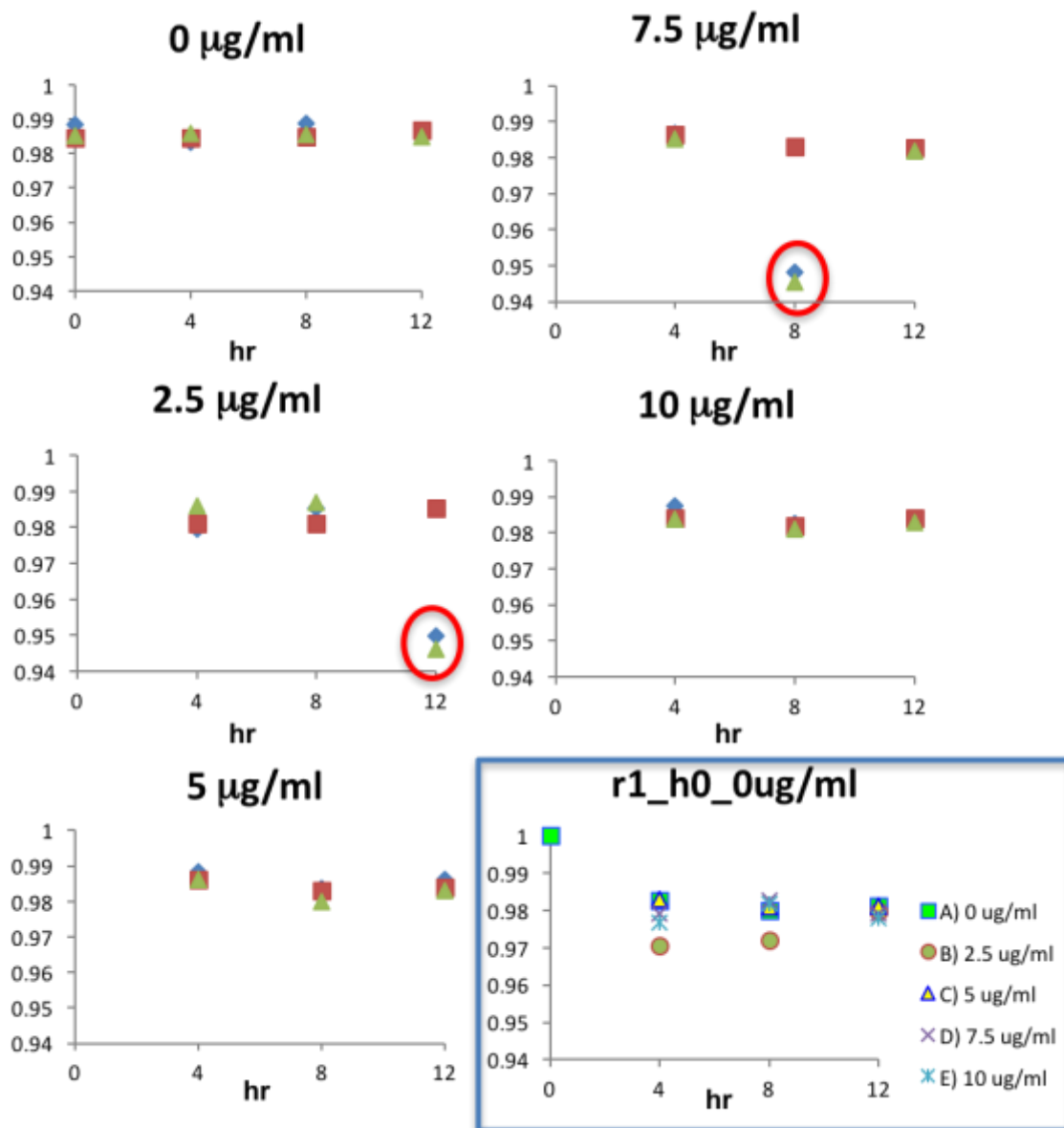
Fig. 2.19: **Quality of RNA samples for RNAseq analysis.** The Spearman's correlation between two biological replicates is a measure of the similarity between two samples. We plotted the correlation between each two of the three biological replicates in each treatment (as defined by concentration and time). Blue diamonds, red squares and green triangles represent the correlations between replicates 1 and

2, 1 and 3, and 2 and 3, respectively. Most of correlation between the two biological replicates at each time point are more than 0.98, except for two samples (bio_rep2 treated with 7.5 mg/ml at 8 hr and bio_rep2 treated with 2.5 mg/ml at 12 hr). These samples exhibited lower correlations (around 0.95) with the other two replicates (bio_rep2 vs bio_rep1: blue diamond and bio_rep2 vs bio_rep3: green triangle).

The overall difference between the samples at different time points is rather small, as seen in the highlighted blue box. In fact, the correlation between any two samples of different concentration or different time is always higher than 0.95. Therefore, we removed the samples that exhibited correlations lower than 0.95 from further analysis.

A = 0 µg/ml curcumin
B = 2.5 µg/ml curcumin
C = 5.0 µg/ml curcumin
D = 7.5 µg/ml curcumin
E = 10.0 µg/ml curcumin
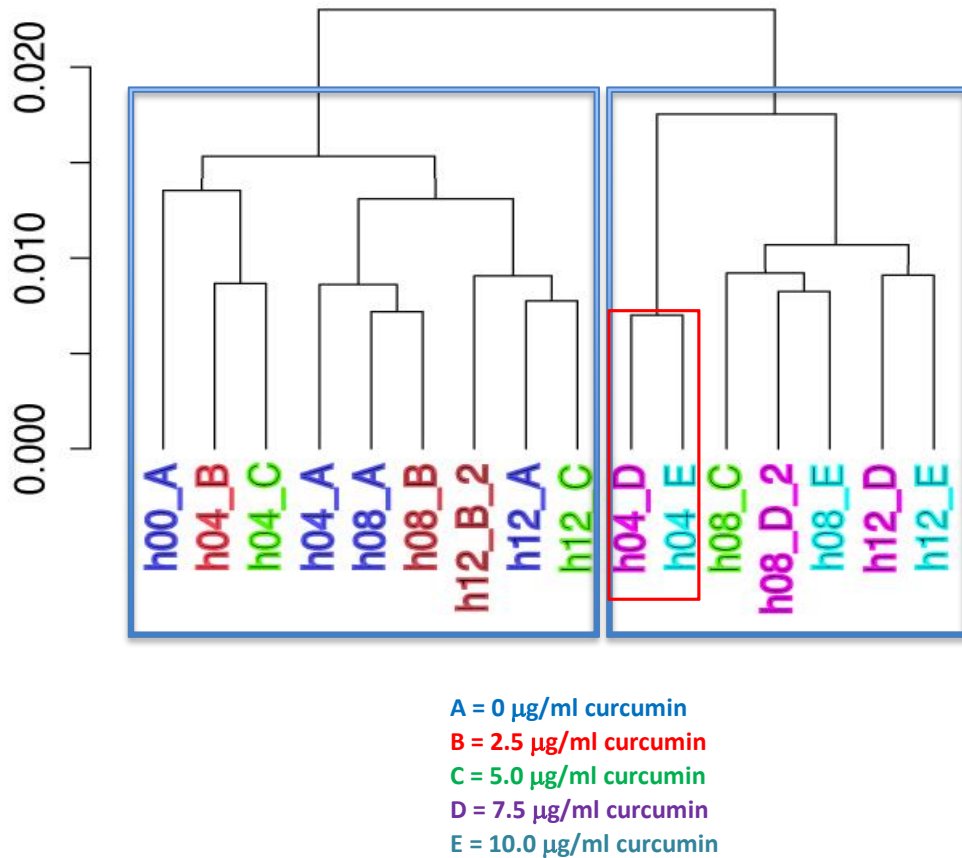
Fig. 2.20: Hierarchical clustering analysis of RNAseq samples. We averaged the values of the replicate RNA-seq samples at each time point and each curcumin concentration as indicated and calculated the distance (Spearman's correlation) between the samples. The hierarchical clustering dendrogram illustrates the relationships between the samples based on these distance calculations. In the dendrogram, each leaf represents one condition (time and curcumin concentration) and the vertical distances between the joints represent the dissimilarity between the samples (see scale on the left, arbitrary units). The two blue boxes indicate the two most dissimilar groups.

the 0, 2.5 and 5 µg/ml samples and the 7.5 and 10 µg/ml samples. These data support

the cell proliferation phenotypes we observed in Figs. 2.1 and 2.10 where there was

little effect on cell proliferation at concentrations below 7.5 µg/ml curcumin.   In

addition, Fig. 2.20 shows that time is a factor in the effect of curcumin but that

concentration is the primary factor.

XVIII. Multidimensional Scaling (MDS)

This approach provides another method to examine the data using a different

computational approach (Rosengarten et al., 2015). The transcriptome of each strain

can be thought of as a single point in a multidimensional space, where each gene

defines a dimension and each mRNA abundance value determines the position of the

point in that dimension. An MDS plot provides a two-dimensional view that is the

best representation of the multidimensional distances between the points. When two

points in the graph are close, it means that the transcriptomes of the two samples are

similar to one another.   Figure 2.21 shows that this analysis provides the same

interpretation as was seen by hierarchical clustering analysis (Fig. 2.20) where there

is a clear concentration threshold of the effect of curcumin on transcription in

proliferating *D. discoideum* cells.   Again, time has less of an effect.   This analysis

validates the analysis provided by the hierarchical clustering method.
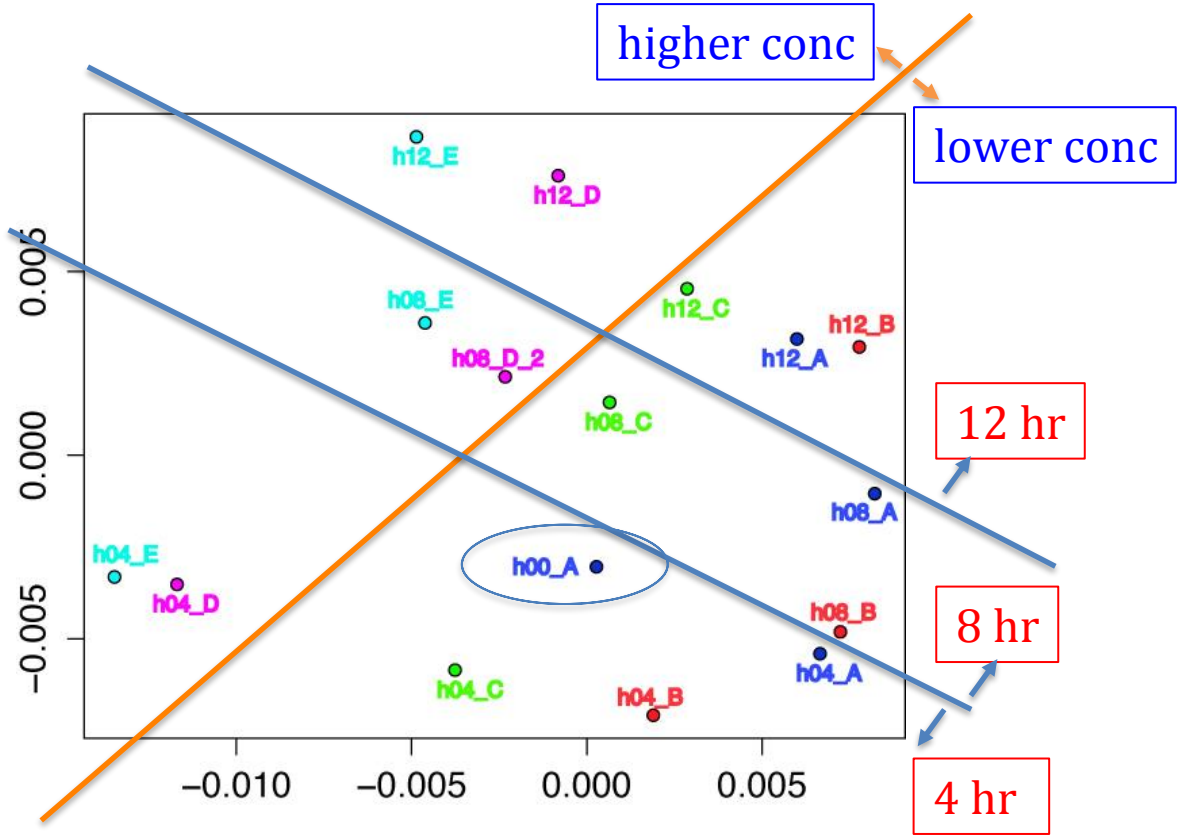
Fig. 2.21: Multidimensional scaling analysis of RNAseq samples. The distances calculated in Fig. 2.20 are shown as a multidimensional scaling (MDS) plot. Each point on the graph represents a sample (time and curcumin concentration, as indicated) and the distances between the points represent the dissimilarity between them – the closer two point are, the more similar they are. The blue lines indicate groups that can be visually separated according to time and the orange line shows groups that can be visually separated by curcumin concentration. The axes units are arbitrary.

XIX. Curcumin has an early transient effect on gene expression in *D. discoideum*.

Fig. 2.22 A shows the comparison of the transcriptional profiles of the 4 hour samples with 0 and 10 μg/ml curcumin. The aim was to look at an early time point using a concentration of curcumin which the earlier analysis indicated has a robust effect on transcription, and where there would be a minimum of unrelated effects that accumulate in cells with time. Fig. 2.22 B presents the Bay Seq differential expression analysis which shows a dose-dependent early change in gene expression where 678 total genes were effected – 533 genes were up-regulated and 145 were down-regulated. The analysis shows that the early change in gene expression is transient and that these up-regulated mRNAs do not accumulate with time. Fig. 2.22 C shows the Bay Seq analysis of a more refined stringency where only genes showing a minimum 3-fold change in differential expression were examined which limited the numbers to 192 genes up-regulated and 39 down-regulated. This analysis shows the same early, transient transcriptional response as the previous analysis using the larger set of affected genes. The overall conclusion is that there is a limited set of specific early response genes that respond to curcumin in a transient manner.

XX. Gene ontology (GO) term enrichment analysis reveals molecular pathways associated with the early response to curcumin.

Gene ontology term enrichment analysis categorizes the individual changes in the transcriptome by molecular factor, biological process and cell compartment. As seen in Table 2.6 A, the analysis of the 533 genes up-regulated in the first 4 hours of

Fig. 2.22: Comparison of transcriptional profiles of 4 hour samples with 0 and 10 µg/ml curcumin. We analyzed the differential expression (using baySeq) between the 4h samples treated with 0 and 10 µg/ml curcumin as illustrated in (A). We then projected the expression patterns of the differentially expressed genes we discovered as yellow-blue heat maps to illustrate their expression patterns in all the time points and all the curcumin concentrations as indicated below the columns (B). In the heat maps, each row represents the abundance levels of one transcript (scale indicated above the chart) and each column represents one condition (time and curcumin concentration). Transcripts that exhibited increased abundance with increased curcumin concentration are clustered above the orange line (up) and increased curcumin concentration are clustered above the orange line (up) and

transcripts that exhibited reduced abundance are clustered below (down). The number of genes in each cluster is indicated. We show all the genes we found in B and a selected subset that showed at least a 3-fold change in C.

Term	Annotate	Signif	Expect	Classic	fold_enrich	categ	genes
oxidoreductase activity	513	50	24.4	6.00E-07	2.0	MF	aifB,alrE,chdh,cxgS,cyp508A4,cyp515B1,cyp515B1,iliB,malA,maoB-1,msrA,noxB,rnrA
hydrolase activity, acting on acid anhyd…	94	14	4.47	0.00013	3.1	MF	abcA2,abcB1,abcC12,abcC3,abcG11,abcG12,abcG14,abcG15,abcG17-1,abcG21,abcG9
phospholipid binding	49	8	2.33	0.00197	3.4	MF	dagA,hipA,nxnA,rtoA,tom1,vps5
protein homodimerization activity	29	6	1.38	0.00208	4.3	MF	abpC,csaA,dimB,dstC,mhcA,pefA
alpha,alpha-trehalose-phosphate synthase…	2	2	0.1	0.00226	20.0	MF	tpsA,tpsB
NAPE-specific phospholipase D activity	7	3	0.33	0.00324	9.1	MF	DDB_G0271270,DDB_G029392,pldB
coenzyme binding	123	13	5.85	0.00547	2.2	MF	aifB,chdh,malA,noxB,padA,sir2B
UDP-glucosyltransferase activity	10	3	0.48	0.00997	6.3	MF	tpsA,tpsB,ugt52
response to osmotic stress	109	22	5.1	4.10E-09	4.3	BP	abcB1,abcG21,abpC,act1,act19,cprA,cupA,cupB,cupC,cupF,cupG,cupH,cupI,dstC,dymB,mhcA,p2xC,rtoA,sig1
response to salt stress	**13**	**8**	**0.61**	**2.20E-08**	**13.1**	**BP**	**cupA,cupB,cupC,cupF,cupG,cupH,cupI,dstC**
oxidation-reduction process	561	52	26.23	1.10E-06	2.0	BP	aco2,aifB,alrE,chdh,cshA,cxgS,cyp508A4,cyp515B1,cyp516B1,iliB,malA,maoB-1,msrA,noxB,rnrA
hyperosmotic response	57	12	2.67	9.50E-06	4.5	BP	abcB1,abcG21,abpC,act1,act19,cprA,DDB_G0267924,dstC,rtoA,sig1
response to heat	11	4	0.51	0.00119	7.8	BP	DDB_G0291314,dnaja1,dstC,rtoA
regulation of cell cycle process	37	7	1.73	0.00141	4.0	BP	aurK,bub1,cdc45,kif13,ube2c
steroid metabolic process	28	6	1.31	0.00157	4.6	BP	cas1,DDB_G0270946,erg2,ugt52
spindle organization	16	4	0.75	0.00547	5.3	BP	aurK,icpA,kif13,kif2
regulation of mitotic cell cycle phase t…	16	4	0.75	0.00547	5.3	BP	bub1,cdc45,DDB_G0273201,ube2c
oligosaccharide biosynthetic process	3	2	0.14	0.00634	14.3	BP	tpsA,tpsB
DNA replication initiation	10	3	0.47	0.00951	6.4	BP	cdc45,mcm4,polA1
spindle midzone	**2**	**2**	**0.09**	**0.0022**	**22.2**	**CC**	**aurK,icpA**
spindle pole	10	3	0.47	0.0097	6.4	CC	aurK,icpA,plk

Table 2.6 A: Gene ontology enrichment of up-regulated gene set (533 genes) upon early exposure to curcumin. Genes identified are involved in *D. discoideum* oxidoreductase activity, response to osmotic, salt, heat stress and the cell cycle.

treatment, there are interesting clusters of genes. Of particular interest are the groups of genes encoding oxidoreductase enzymes, osmotic/salt/heat stress enzymes, and cell cycle related proteins. These data reemphasize the complex pleiotropic cellular response to curcumin.

The GO analysis of the genes down-regulated in the first 4 hours of curcumin treatment provides an interesting counterpoint to the up-regulated genes (Table 2.6 B). The down-regulated groups include genes encoding proteins involved in apoptosis and cell death, defense to bacteria, and peroxisome function. These data indicated that cells down-regulate their cell death mechanisms in response to curcumin as a survival mechanism. It is notable that genes involved in sphingomyelin catabolism are also down-regulated, because of reports that sphingolipids are involved in regulating the cellular response to curcumin (Moussavi et al., 2006; Shakor et al., 2014; Yang et al., 2012).

XXI. The early effect on transcription is replaced with a unique transcriptional profile after extended exposure to curcumin.

The BaySeq analysis of the 4-hour time points indicated a robust and transient early transcriptional response to curcumin. This raised the question whether there was any subsequent transcriptional response following the early response. To address this question, the 12-hour time points comparing untreated cells to those treated with 10 μg/ml were analyzed (Fig. 2.23 A). In contrast to the earlier analysis of the 4 hour samples, the samples from the later time point have more genes that are down-

Term	Annotate	Signif	Expect	Classic	fold_enrich	categ	genes
deoxyribonuclease II activity	11	3	0.16	0.00044	18.8	MF	DDB_G0270850,DDB_G0270958,dnase2
hydrolase activity, acting on glycosyl b…	103	7	1.49	0.00067	4.7	MF	alyD-1,athl1,DDB_G0274181,,sgmA,sgmB
lysozyme activity	13	3	0.19	0.00075	15.8	MF	alyD-1,DDB_G0274181,DDB_G0293566
sphingomyelin phosphodiesterase activity	5	2	0.07	0.002	28.6	MF	sgmA,sgmB
phospholipase activity	22	3	0.32	0.00368	9.4	MF	plbE,sgmA,sgmB
thiolester hydrolase activity	9	2	0.13	0.00695	15.4	MF	ppt2,ppt3
transporter activity	339	11	4.89	0.00935	2.2	MF	abcA7,abcA8,,fhbA,mcfZ,nramp1,pkd2
galactose metabolic process	6	4	0.09	7.50E-07	44.4	BP	DDB_G0282525,galE,galK,uppA
pantothenate metabolic process	2	2	0.03	0.00023	66.7	BP	DDB_G0286637,panC
apoptotic DNA fragmentation	11	3	0.17	0.00052	17.6	BP	DDB_G0270850,DDB_G0270958,dnase2
peptidoglycan metabolic process	14	3	0.21	0.00111	14.3	BP	alyD-1,DDB_G0274181,DDB_G0293566
L-serine biosynthetic process	4	2	0.06	0.00135	33.3	BP	serA,serC
sphingomyelin catabolic process	4	2	0.06	0.00135	33.3	BP	sgmA,sgmB
defense response to bacterium	23	3	0.35	0.00488	8.6	BP	alyD-1,DDB_G0274181,DDB_G0293566
cofactor biosynthetic process	73	5	1.11	0.00495	4.5	BP	coq7,DDB_G0286637,DDB_G0290401,hemC,panC
organic anion transport	51	4	0.78	0.00736	5.1	BP	DDB_G0284051,DDB_G0287303,DDB_G0292424,mcfZ
glucosamine-containing compound metaboli…	10	2	0.15	0.00955	13.3	BP	DDB_G0287465,nagB1
peroxisomal membrane	22	3	0.33	0.00409	9.1	CC	ddo-2,mcfQ,pex13
microbody	50	4	0.75	0.00645	5.3	CC	DDB_G0277477,ddo-2,mcfQ,pex13

Table 2.6 B: Gene ontology of down regulated genes (145 genes) upon early exposure to curcumin. Genes identified are involved in *D. discoideum* apoptosis, cell death, defense response to bacterium and peroxisome/microbody.
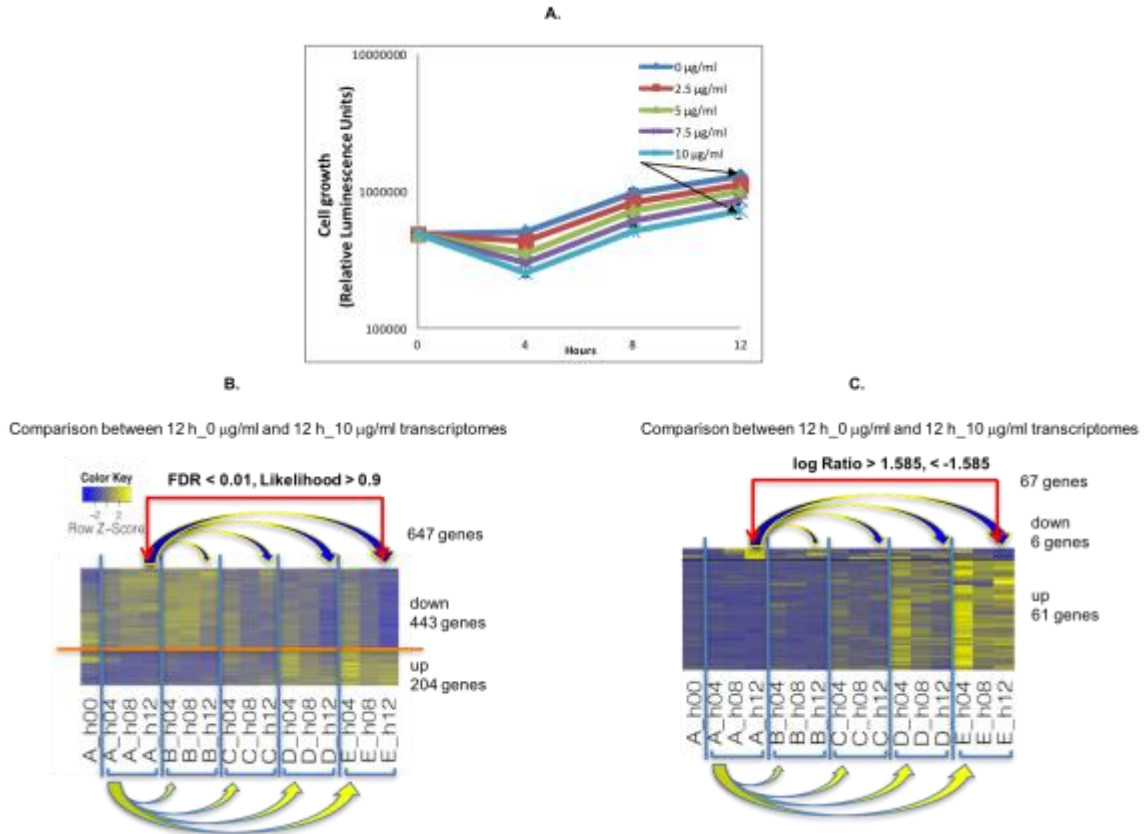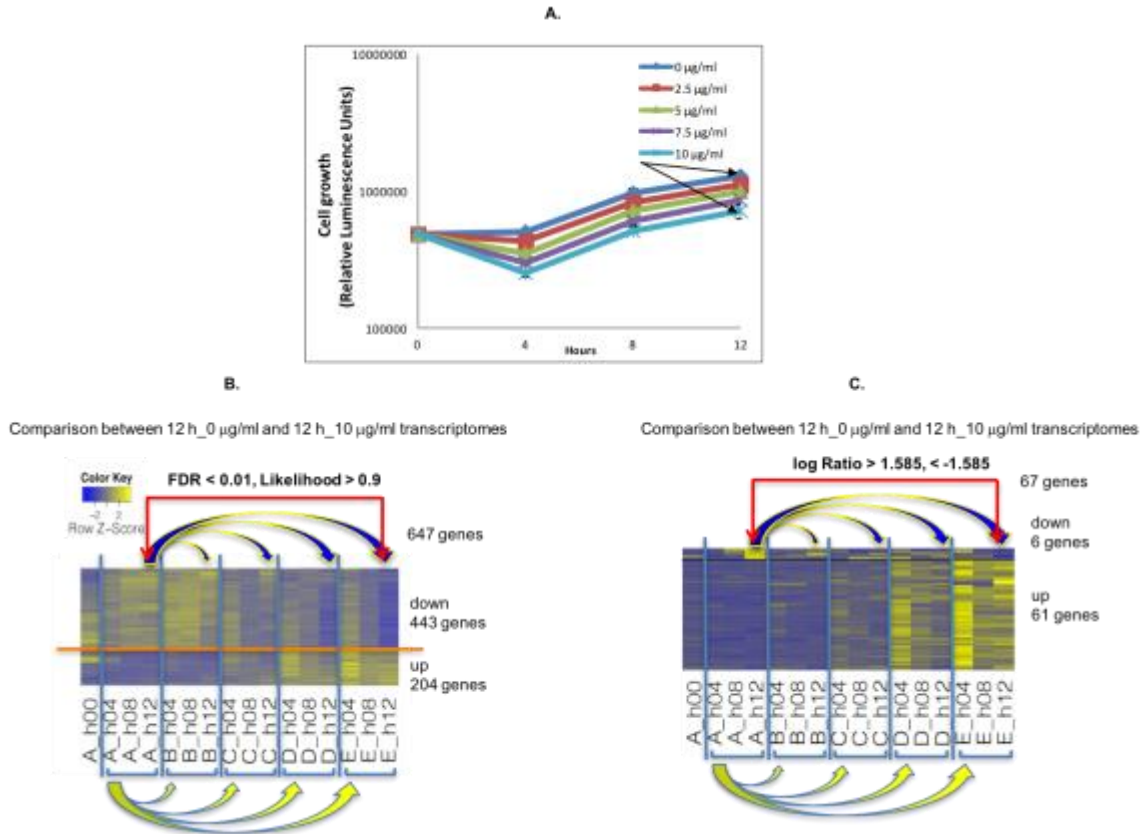
Fig. 2.23: Comparison of transcriptional profiles of 12 hour samples with 0 and 10 µg/ml curcumin. We analyzed the differential expression (using BaySeq) between the 12h samples treated with 0 and 10 µg/ml curcumin as illustrated in (A). We then projected the expression patterns of the differentially expressed genes we discovered as yellow-blue heat maps to illustrate their expression patterns in all the time points and all the curcumin concentrations as indicated below the columns (B). In the heat maps, each row represents the abundance levels of one transcript (scale indicated above the chart) and each column represents one condition (time and curcumin concentration). Transcripts that exhibited increased abundance with increased curcumin concentration are clustered above the orange line (up) and increased curcumin concentration are clustered above the orange line (up) and

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transcripts that exhibited reduced abundance are clustered below (down). The number of genes in each cluster is indicated. We show all the genes we found in B and a selected subset that showed at least a 3-fold change in C.

regulated (443) than are up-regulated (204) (Fig. 2.23 B). However, when the stringency is increased to examine only those genes showing a 3-fold change in transcription the number of down-regulated genes drops precipitously to 6 (1.4%) while the up-regulated genes only decrease to 61 (30%) (Fig. 2.23 C). Of these stringently selected genes 3 of the down-regulated genes overlapped with the 4-hour BaySeq up-regulated list, while 51 of the up-regulated genes overlapped with the 4-hour BaySeq down-regulated list. These data indicate that there is a unique and increasingly limited transcriptional response to curcumin that takes place after the robust initial early response seen at 4 hours.

XXII. GO term enrichment analysis also reveals unique molecular pathways associated with extended exposure to curcumin.

GO term enrichment analysis of the later 12-hour samples (Table 2.7 A) indicates that up-regulated genes encoding proteins involved in oxidoreductase activity, antioxidant activity, vitamin binding and the response to abiotic stimuli are affected. Some of these genes, (e.g., the oxidoreductase genes) are also up-regulated in the early 4-hour samples, but many others are uniquely up regulated at this time. In contrast, the down-regulated genes of the 12-hour samples (Table 2.7 B) encode proteins involved with cell cycle control, DNA replication, responses to drugs, and oxidoreductase activity. Overall, the GO term enrichment lists at 12 hours widely differ from those at 4 hours and confirm the analyses of the transcriptomes as a whole (Figs. 2.20 and 2.21).

Term	Annotated	Significant	Expected	classic	fold_enrich	categ	genes
oxidoreductase activity	513	33	10.19	8.10E-10	3.2	MF	aifB,ccs,cyp508A4,cyp516B1, DDB_G genes, hpdl-1,ndrJ,scdA,sqrdl
ATPase activity, coupled to transmembran...	92	8	1.83	0.00045	4.4	MF	abcB1,abcG11,abcG12,abcG15,abcG17-1,abcG21,abcG9,patA
nucleic acid binding transcription facto...	66	6	1.31	0.00188	4.6	MF	bzpP,dimB,dstB,dstC,gtaE,stkA
sequence-specific DNA binding transcript...	66	6	1.31	0.00188	4.6	MF	bzpP,dimB,dstB,dstC,gtaE,stkA
cobalamin binding	4	2	0.08	0.00229	25.0	MF	mut,ndrJ
antioxidant activity	32	4	0.64	0.00348	6.3	MF	ccs,DDB_G0268192,DDB_G0269270,DDB_G0272280
transmembrane transporter activity	263	12	5.22	0.00589	2.3	MF	abcB1,abcG11,abcG12,abcG15,abcG17-1,abcG21,abcG9,DDB_G0271672,DDB_G0283629,patA,rhgB,symA
vitamin binding	20	3	0.4	0.00682	7.5	MF	DDB_G0287671,mut,ndrJ
transition metal ion binding	575	20	11.42	0.00933	1.8	MF	ccs,cyp508A4,cyp516B1,DDB_G0268322,DDB_G0271270,DDB_G0271294,DDB_G0271740,DDB_G0272184,DDB_G0273077,DDB_G0276037,DDB_G0279843,DDB_G0286305,DDB_G0287671,DDB_G0292096,DDB_G0292642,DDB_G0293328,gtaE,sf1,stkA,zfand
oxidation-reduction process	561	33	10.87	4.70E-09	3.0	BP	aifB,ccs,cyp508A4,cyp516B1, DDB_G genes, hpdl-1,ndrJ,scdA,sqrdl
single-organism metabolic process	1305	43	25.28	0.00018	1.7	BP	aifB,asns,ccs,cyp508A4,cyp516B1, DDB_G genes, gcsA,gxcF,hpdl-1,maspS,mgp1,ndrJ,scdA,sqrdl
transmembrane transport	304	13	5.89	0.00592	2.2	BP	abcB1,abcG11,abcG12,abcG15,abcG17-1,abcG21,abcG9,DDB_G0271672,DDB_G0283629,mcfV,patA,rhgB,symA
response to abiotic stimulus	143	8	2.77	0.00645	2.9	BP	abcB1,abcG21,dnaja1,dstC,dymB,mgp1,phgA,sigJ
regulation of cell proliferation	8	2	0.15	0.00967	13.3	BP	DDB_G0267706,dstB
contractile vacuole	47	5	0.89	0.0018	5.6	CC	dymB,hipA,mgp1,patA,phgA

Table 2.7 A: GO analysis of upregulated genes upon extended exposure to curcumin. Genes upregulated in *D. discoideum* upon extended exposure to curcumin are involved in oxidoreductase activity, antioxidant activity, vitamin binding and response to abiotic stimulus.

Term	Annotated	Significant	Expected	classic	fold_enrich	categ	genes
hydrolase activity, hydrolyzing O-glycos...	86	13	3.67	6.40E-05	3.5	MF	chid1,ctbsA,ctbsB,..DDB_G genes,,ilG,parG,rlF
DNA primase activity	**3**	**3**	**0.13**	**7.70E-05**	**23.1**	**MF**	**polA1,polA3,polA4**
deaminase activity	12	5	0.51	8.50E-05	9.8	MF	ada,cda,DDB_G0275179,ftcd,guaD
chitin binding	8	4	0.34	0.0002	11.8	MF	chid1,ctbsA,ctbsB,DDB_G0268090
oxidoreductase activity, acting on paire...	**20**	**5**	**0.85**	**0.00126**	**5.9**	**MF**	**cyp508A2-1,cyp508A3-1,cyp508B1,cyp518A1,cyp519E1**
scavenger receptor activity	2	2	0.09	0.00182	22.2	MF	DDB_G0284479,dduF
oxidoreductase activity, acting on paire...	86	10	3.67	0.00346	2.7	MF	cyp508A2-1,cyp508A3-1,cyp508B1,cyp513A1,cyp513A3,cyp513C1,cyp518A1,cyp519E1,DDB_G0274231,fmoA
ammonia-lyase activity	8	3	0.34	0.00368	8.8	MF	ftcd,hal,sds
DNA replication origin binding	**3**	**2**	**0.13**	**0.0053**	**15.4**	**MF**	**cdc45,mcm5**
carbon-nitrogen lyase activity	11	3	0.47	0.00986	6.4	MF	ftcd,hal,sds
DNA replication, synthesis of RNA primer	**3**	**3**	**0.14**	**9.40E-05**	**21.4**	**BP**	**polA1,polA3,polA4**
receptor-mediated endocytosis	**7**	**4**	**0.32**	**0.00013**	**12.5**	**BP**	**adcB,adcC,DDB_G0284479,dduF**
response to drug	27	7	1.23	0.00015	5.7	BP	crtp3,cyp508A2-1,cyp508A3-1,cyp508B1,cyp518A1,cyp519E1,fps
chromosome segregation	38	8	1.73	0.00025	4.6	BP	icpA,kif13,kif2,ncapGa,nek2,smc1,smc3,top2
desensitization of G-protein coupled rec...	4	3	0.18	0.00036	16.7	BP	adcB,adcC,carA-1
DNA replication initiation	**10**	**4**	**0.46**	**0.00071**	**8.7**	**BP**	**cdc45,gins4,mcm5,polA1**
sister chromatid segregation	18	5	0.82	0.001	6.1	BP	kif2,ncapGa,smc1,smc3,top2
regulation of cell cycle process	37	7	1.69	0.00121	4.1	BP	aurK,bub1,cdc45,DDB_G0273201,DDB_G0291842,kif13,top2
exogenous drug catabolic process	**20**	**5**	**0.91**	**0.00168**	**5.5**	**BP**	**cyp508A2-1,cyp508A3-1,cyp508B1,cyp518A1,cyp519E1**
G-protein coupled receptor internalizati...	**2**	**2**	**0.09**	**0.00207**	**22.2**	**BP**	**adcB,adcC**
mitotic sister chromatid segregation	16	4	0.73	0.00498	5.5	BP	kif2,ncapGa,smc1,smc3
spindle organization	16	4	0.73	0.00498	5.5	BP	aurK,icpA,kif13,kif2
double-strand break repair via break-ind...	**3**	**2**	**0.14**	**0.00602**	**14.3**	**BP**	**cdc45,gins4**
mitotic cell cycle phase transition	19	4	0.87	0.00952	4.6	BP	bub1,cdc45,DDB_G0273201,top2
nuclear replication fork	**10**	**5**	**0.43**	**3.00E-05**	**11.6**	**CC**	**cdc45,gins4,polA1,polA3,polA4**
alpha DNA polymerase:primase complex	**3**	**3**	**0.13**	**7.90E-05**	**23.1**	**CC**	**polA1,polA3,polA4**
condensed chromosome	29	6	1.25	0.00124	4.8	CC	aurK,DDB_G0273201,icpA,ncapGa,smc1,smc3
cohesin complex	2	2	0.09	0.00185	22.2	CC	smc1,smc3
spindle midzone	**2**	**2**	**0.09**	**0.00185**	**22.2**	**CC**	**aurK,icpA**
primosome complex	**2**	**2**	**0.09**	**0.00185**	**22.2**	**CC**	**polA3,polA4**
microtubule associated complex	29	5	1.25	0.00725	4.0	CC	aurK,icpA,kif13,kif2,kif5

Table 2.7 B: GO analysis of down-regulated genes upon extended exposure to curcumin. Genes down-regulated in *D. discoideum* upon extended exposure to curcumin encode proteins involved with cell cycle control, DNA replication, responses to drugs, and oxidoreductase activity upon extended exposure to curcumin.

XXIII. BaySeq analysis of gene expression comparing the 4 and 12-hour transcription profiles.

This increasingly refined analysis was done taking into consideration that background changes in gene expression accompany cell growth that have nothing to do with the effect of curcumin. To address this issue the genes that showed altered gene expression in the absence of curcumin, i.e., those that change due to the normal physiology of dividing cells, were subtracted from the analysis (Fig. 2.24). Genes that are up-regulated in the absence of curcumin are shown in Table 2.8 and are predictably involved with various cell cycle associated functions. The equivalent analysis of down-regulated genes in the absence of curcumin revealed only 7 genes (data not shown). Tables 2.9 and 2.10 list the genes that are down-regulated (773) and up-regulated (161) respectively after omitting the changes that were independent of curcumin (Table 2.8).

XXIV. Antioxidant gene response

The RNAseq data were examined to determine the effect on the transcription of the *cat*A, *sod*A, *sod*B and *sod*2 genes. Consistent with what we had seen by RT_PCR analysis (Fig 2.4), the RNA levels in cells treated with curcumin were slowly trending down at 12 hours (Fig 2.25).
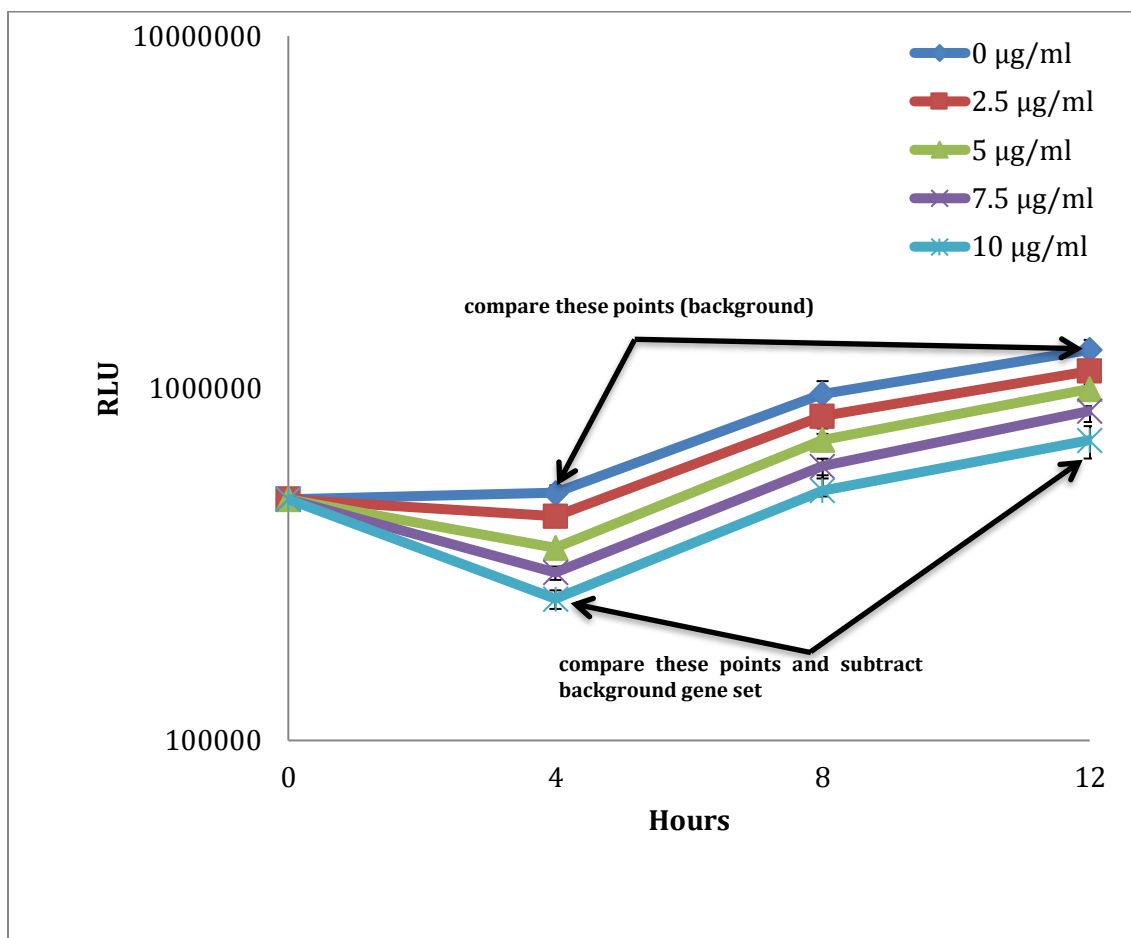
Fig. 2.24: Analyses of transcriptional profiles due to curcumin treatment. Differential expression (using BaySeq) analysis were done taking into account the many background changes in gene expression as a result of normal cell physiology changes accompanied with growth. Those changes were subtracted from our analyses.

Term	Annotated	Significant	Expected	classic	fold_enrich	categ	genes
DNA-directed DNA polymerase activity	19	2	0.08	0.0031	25.0	MF	polA1,polA2
microtubule binding	24	2	0.11	0.0049	18.2	MF	DDB_G0280249,kif13
DNA helicase activity	29	2	0.13	0.0072	15.4	MF	cdc45,wrnip1
ubiquitin protein ligase binding	**30**	**2**	**0.13**	**0.0076**	**15.4**	**MF**	**DDB_G0288381,DDB_G0288697**
tubulin binding	31	2	0.14	0.0081	14.3	MF	DDB_G0280249,kif13
regulation of cell cycle process	**37**	**4**	**0.17**	**2.40E-05**	**23.5**	**BP**	**aurK,bub1,cdc45,kif13**
protein K63-linked ubiquitination	**5**	**2**	**0.02**	**0.00021**	**100.0**	**BP**	**DDB_G0288381,DDB_G0288697**
mitosis	68	4	0.32	0.00026	12.5	BP	bub1,cdc20,kif13,smc2
mitotic cell cycle	200	6	0.94	0.00029	6.4	BP	aurK,bub1,cdc20,cdc45,kif13,smc2
DNA replication	78	4	0.36	0.00045	11.1	BP	cdc45,polA1,polA2,wrnip1
cell division	156	5	0.73	0.00072	6.8	BP	aurK,cdc20,DDB_G0280249,kif13,smc2
DNA replication initiation	**10**	**2**	**0.05**	**0.00093**	**40.0**	**BP**	**cdc45,polA1**
microtubule cytoskeleton organization	42	3	0.2	0.00095	15.0	BP	aurK,DDB_G0280249,kif13
postreplication repair	11	2	0.05	0.00114	40.0	BP	DDB_G0288381,DDB_G0288697
mitotic spindle organization	12	2	0.06	0.00136	33.3	BP	aurK,kif13
spindle organization	**16**	**2**	**0.07**	**0.00245**	**28.6**	**BP**	**aurK,kif13**
regulation of mitotic cell cycle phase t...	**16**	**2**	**0.07**	**0.00245**	**28.6**	**BP**	**bub1,cdc45**
protein polyubiquitination	19	2	0.09	0.00346	22.2	BP	DDB_G0288381,DDB_G0288697
mitotic cell cycle phase transition	19	2	0.09	0.00346	22.2	BP	bub1,cdc45
chromosomal part	**104**	**5**	**0.48**	**9.90E-05**	**10.4**	**CC**	**aurK,cdc45,DDB_G0293620,polA1,smc2**
nuclear replication fork	**10**	**2**	**0.05**	**0.00091**	**40.0**	**CC**	**cdc45,polA1**
nuclear chromosome part	48	3	0.22	0.00134	13.6	CC	aurK,cdc45,polA1
replication fork	16	2	0.07	0.00238	28.6	CC	cdc45,polA1
chromosome, centromeric region	22	2	0.1	0.00451	20.0	CC	aurK,DDB_G0293620
condensed chromosome	29	2	0.13	0.00776	15.4	CC	aurK,smc2

Table 2.8: GO analysis of up-regulated genes without exposure to curcumin.

Genes up-regulated in *D. discoideum* without exposure to curcumin are mostly cell cycle related genes.

Term	Annotated	Significant	Expected	classic	fold_enrich	categ	genes
DNA primase activity	**3**	**3**	**0.2**	**0.00031**	**15.0**	**MF**	**polA1,polA3,polA4**
purinergic nucleotide receptor activity	13	5	0.88	0.00116	5.7	MF	carA-1,crlC,p2xA,p2xB,p2xE
intramolecular oxidoreductase activity, ...	**4**	**3**	**0.27**	**0.00119**	**11.1**	**MF**	**ebp,ech1,erg2**
DNA helicase activity	29	7	1.97	0.0027	3.6	MF	cdc45,gins1,gins4,mcm4,mcm5,mcm7,wrnip1
O-acyltransferase activity	5	3	0.34	0.00282	8.8	MF	DDB_G0276523,DDB_G0282197,dgat1
scavenger receptor activity	2	2	0.14	0.00461	14.3	MF	DDB_G0284479,dduF
fucosidase activity	2	2	0.14	0.00461	14.3	MF	alfA,DDB_G0276661
calcium-release channel activity	6	3	0.41	0.00535	7.3	MF	p2xA,p2xB,p2xE
oxidoreductase activity, acting on paire...	20	5	1.36	0.00939	3.7	MF	cyp508A4,cyp515B1,cyp516B1,cyp518A1,cyp519E1
mitosis	68	20	4.78	2.10E-08	4.2	BP	anapc10,anapc3,anapc7,bub1,bub3,cdc20,cdk1,dcc1,DDB_G0273201,icpA,kif2,kif4,ncapD3,ncapGa,pds5,plk,smc1,smc2,smc3,ube2c
DNA strand elongation involved in DNA re...	**14**	**8**	**0.98**	**1.20E-06**	**8.2**	**BP**	**DDB_G0283189,lig1,polA1,repG,rfc2,rfc3,rfc4,rfc5**
leading strand elongation	8	6	0.56	2.90E-06	10.7	BP	DDB_G0283189,polA1,rfc2,rfc3,rfc4,rfc5
sister chromatid segregation	**18**	**8**	**1.26**	**1.30E-05**	**6.3**	**BP**	**dcc1,kif2,ncapD3,ncapGa,smc1,smc2,smc3,top2**
DNA replication initiation	10	6	0.7	1.90E-05	8.6	BP	cdc45,gins4,mcm4,mcm5,mcm7,polA1
mitotic sister chromatid segregation	16	7	1.12	5.30E-05	6.3	BP	dcc1,kif2,ncapD3,ncapGa,smc1,smc2,smc3
response to salt stress	13	6	0.91	0.00013	6.6	BP	cupB,cupC,cupF,cupG,cupI,dstC
calcium ion transport	14	6	0.98	0.00022	6.1	BP	DDB_G0284777,DDB_G0289473,p2xA,p2xB,p2xE,regA
DNA replication, synthesis of RNA primer	**3**	**3**	**0.21**	**0.00035**	**14.3**	**BP**	**polA1,polA3,polA4**
regulation of mitotic cell cycle phase t...	16	6	1.12	0.00051	5.4	BP	anapc10,bub1,cdc45,DDB_G0273201,top2,ube2c
mitotic sister chromatid cohesion	**4**	**3**	**0.28**	**0.00131**	**10.7**	**BP**	**dcc1,smc1,smc3**
deoxyribonucleotide metabolic process	8	4	0.56	0.00134	7.1	BP	dak,DDB_G0293580,dut,rnrA
mitotic cell cycle phase transition	19	6	1.34	0.00144	4.5	BP	anapc10,bub1,cdc45,DDB_G0273201,top2,ube2c
hypoxanthine salvage	2	2	0.14	0.00493	14.3	BP	ada,DDB_G0275179
DNA biosynthetic process	24	6	1.69	0.00529	3.6	BP	DDB_G0283189,DDB_G0293864,lig1,polA1,polA2,polD2
response to heat	11	4	0.77	0.00534	5.2	BP	DDB_G0282313,dnaja1,dstC,rtoA
calcium ion transmembrane transport	11	4	0.77	0.00534	5.2	BP	DDB_G0289473,p2xA,p2xB,p2xE
response to temperature stimulus	19	5	1.34	0.00856	3.7	BP	cpnA,DDB_G0282313,dnaja1,dstC,rtoA
mitotic chromosome condensation	7	3	0.49	0.00975	6.1	BP	ncapD3,ncapGa,smc2
regulation of calcium-mediated signaling	7	3	0.49	0.00975	6.1	BP	p2xA,p2xB,p2xE
replication fork	**16**	**10**	**1.14**	**1.70E-08**	**8.8**	**CC**	**cdc45,DDB_G0283189,gins4,polA1,polA3,polA4,rfc2,rfc3,rfc4,rfc5**
Elg1 RFC-like complex	**4**	**4**	**0.29**	**2.60E-05**	**13.8**	**CC**	**rfc2,rfc3,rfc4,rfc5**
cell surface	22	8	1.57	8.20E-05	5.1	CC	carA-1,csaA,csbA,csbB,dscA-1,dscD-1,gmsA,sigB
alpha DNA polymerase:primase complex	**3**	**3**	**0.21**	**0.00036**	**14.3**	**CC**	**polA1,polA3,polA4**
cohesin complex	**2**	**2**	**0.14**	**0.00507**	**14.3**	**CC**	**smc1,smc3**
spindle midzone	**2**	**2**	**0.14**	**0.00507**	**14.3**	**CC**	**aurK,icpA**
contractile vacuolar membrane	19	5	1.35	0.00907	3.7	CC	cpnA,p2xA,p2xB,p2xE,vwkA

Table 2.9: Gene ontology enrichment analysis of down regulated gene set only under curcumin treatment. Genes that were down-regulated without curcumin as a result of the cell cycle were omitted in gene ontology enrichment to focus only on genes upregulated as a result of curcumin exposure. Genes identified are involved in oxidoreductase activity and the cell cyce.

Term	Annotated	Significant	Expected	classic	fold_enrich	categ	genes
lipase activity	35	6	0.58	2.10E-05	10.3	MF	DDB_G0277473,DDB_G0291354,plbA,plbE,sgmA,sgmB
oxidoreductase activity, acting on paire...	2	2	0.03	0.00028	66.7	MF	fhbA,fhbB
phospholipase activity	22	4	0.37	0.00043	10.8	MF	plbA,plbE,sgmA,sgmB
glutathione transferase activity	10	3	0.17	0.0005	17.6	MF	DDB_G0272632,DDB_G0280317,mai
caffeoyl-CoA O-methyltransferase activit...	3	2	0.05	0.00082	40.0	MF	omt5,omt6
transition metal ion transmembrane trans...	14	3	0.23	0.00145	13.0	MF	mcfF,nramp1,p80
sphingomyelin phosphodiesterase activity	5	2	0.08	0.00268	25.0	MF	sgmA,sgmB
serine-type exopeptidase activity	6	2	0.1	0.00398	20.0	MF	DDB_G0280105,DDB_G0291912
iron ion transmembrane transporter activ...	7	2	0.12	0.00551	16.7	MF	mcfF,nramp1
pyridoxal phosphate binding	48	4	0.8	0.00819	5.0	MF	aqxt,cysB,hemA,serC
cofactor biosynthetic process	73	11	1.32	6.00E-08	8.3	BP	accA,coq7,coxA,DDB_G0286637,DDB_G0290401,fol1,gchA,hemA,hemF,panC,ptsA
cellular modified amino acid biosyntheti...	19	6	0.34	6.90E-07	17.6	BP	DDB_G0286637,fol1,gchA,panC,pssA,ptsA
vitamin biosynthetic process	8	4	0.14	6.70E-06	28.6	BP	DDB_G0286637,fol1,panC,ptsA
phospholipid catabolic process	11	4	0.2	3.00E-05	20.0	BP	plbA,plbE,sgmA,sgmB
folic acid-containing compound biosynthe...	8	3	0.14	0.0003	21.4	BP	fol1,gchA,ptsA
response to toxic substance	2	2	0.04	0.00032	50.0	BP	fhbA,fhbB
pantothenate metabolic process	2	2	0.04	0.00032	50.0	BP	DDB_G0286637,panC
tetrahydrofolate biosynthetic process	2	2	0.04	0.00032	50.0	BP	fol1,gchA
heme biosynthetic process	10	3	0.18	0.00063	16.7	BP	coxA,hemA,hemF
oxygen transport	3	2	0.05	0.00096	40.0	BP	fhbA,fhbB
folic acid metabolic process	3	2	0.05	0.00096	40.0	BP	fol1,ptsA
response to oxidative stress	69	6	1.24	0.00145	4.8	BP	alrA,aoxA,cysB,DDB_G0275311,gchA,ptsA
L-serine biosynthetic process	4	2	0.07	0.00189	28.6	BP	serA,serC
iron ion transmembrane transport	4	2	0.07	0.00189	28.6	BP	mcfF,nramp1
lipid catabolic process	34	4	0.61	0.00309	6.6	BP	plbA,plbE,sgmA,sgmB
coenzyme A biosynthetic process	6	2	0.11	0.00462	18.2	BP	accA,DDB_G0290401
sphingolipid catabolic process	6	2	0.11	0.00462	18.2	BP	sgmA,sgmB
nucleoside bisphosphate biosynthetic pro...	6	2	0.11	0.00462	18.2	BP	accA,DDB_G0290401
pH elevation	6	2	0.11	0.00462	18.2	BP	ccdc53,myoB
membrane lipid catabolic process	6	2	0.11	0.00462	18.2	BP	sgmA,sgmB
phosphatidylinositol catabolic process	7	2	0.13	0.00639	15.4	BP	plbA,plbE
sulfur compound transport	7	2	0.13	0.00639	15.4	BP	DDB_G0268074,DDB_G0292424
aminoglycan catabolic process	22	3	0.4	0.00688	7.5	BP	alyD-1,DDB_G0287465,DDB_G0293566
serine family amino acid metabolic proce...	22	3	0.4	0.00688	7.5	BP	cysB,serA,serC
sphingolipid biosynthetic process	8	2	0.14	0.00842	14.3	BP	sgmA,sgmB

Table 2.10: Gene ontology enrichment analysis of up-regulated gene set only under curcumin treatment. Genes that were up-regulated without curcumin as a result of the cell cycle were omitted in gene ontology enrichment to focus only on genes upregulated due to curcumin exposure. Genes identified are involved in oxidoreductase activity, response to oxidative stress, pH elevation, phospholipid catabolic process and response to toxic substance.
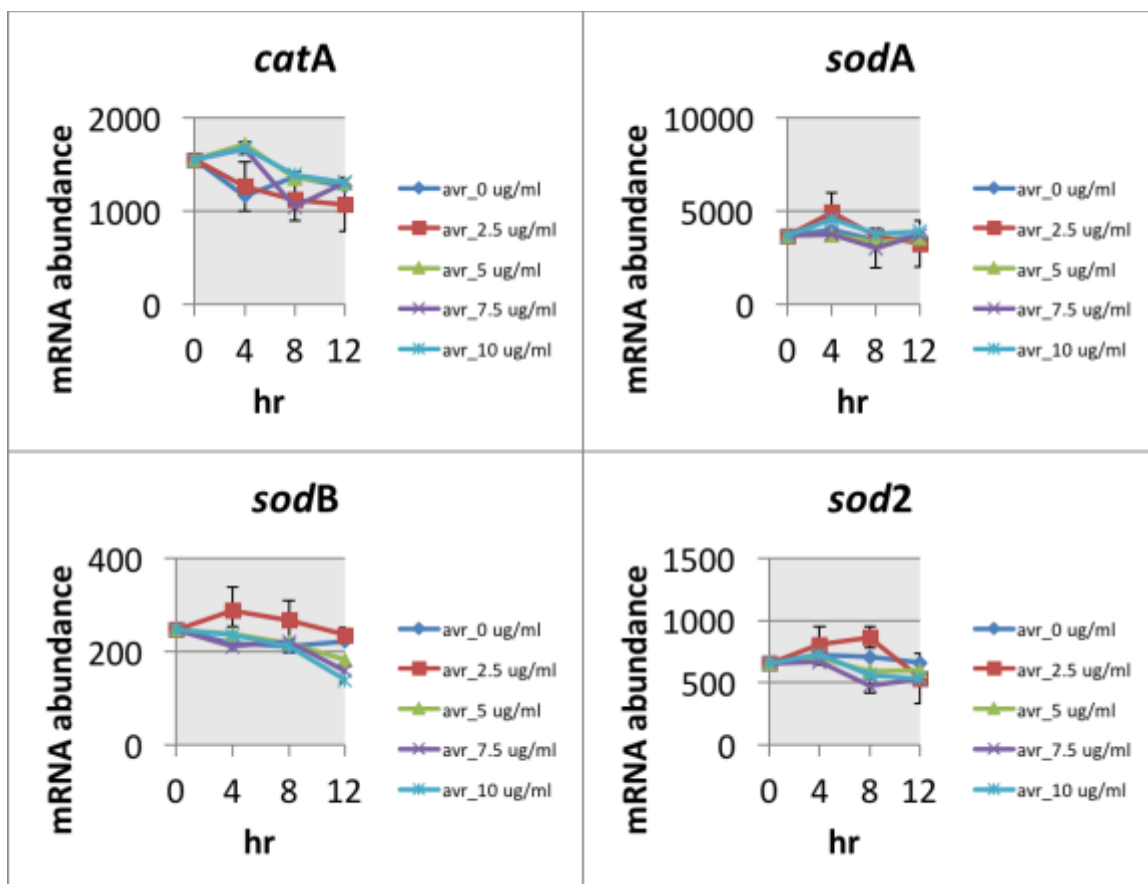
Fig. 2.25: Antioxidant gene response upon curcumin treatment. RNA seq analysis showed a down regulation in *cat*A, *sod*A, *sod*B and *sod*2 by 12 hrs of treatment with curcumin.

E. Discussion

Botanicals are widely used as dietary supplements and for the prevention and treatment of disease. Despite a long history of use, there is generally little evidence supporting the efficacy and safety of these preparations. Curcumin has been used to treat a myriad of human diseases and is widely advertised and marketed for its ability to improve health (Hatcher et al., 2008). The literature on curcumin does not provide a cohesive narrative that suggests how curcumin interacts with cells and affects cell physiology.

In this study we employed *Dictyostelium discoideum* as a lead system to examine the fundamental effects of curcumin on cells, and to begin to parse apart the underlying molecular mechanisms. Studies in *D. discoideum* have previously provided novel insights into the molecular mechanisms which cells use to respond to drugs widely used in human health care (Alexander and Alexander, 2011; Alexander et al., 2006; Williams et al., 2006; Williams, 2005). The current work demonstrates that this system is equally useful for investigations into the fundamental physiological effects of botanicals.

The data demonstrate that curcumin has a profound effect on cell proliferation and an unexpected and pleiotropic effect on gene transcription. Initial experiments on candidate antioxidant genes showed that three superoxide dismutase genes and the catalase A gene were down regulated by curcumin, contrary to what was expected if curcumin was acting directly on the cells as an antioxidant. Curcumin also causes the

cells to increase their production of superoxide. It is unlikely that the increase in superoxide production is the proximal cause of the reduction in anti-oxidant enzyme mRNA levels because 1) the anti-oxidant NAC did not reverse the ability of curcumin to lower the catalase A specific activity nor did it have any effect on cell proliferation, and 2) the pro-oxidants ethidium bromide and menadione both raised the level of catalase specific activity. The conclusion from these observations is that curcumin works through a non-redox mechanism to control mRNA levels of the genes encoding the anti-oxidant enzymes and that the reduction of the enzymes in turn causes the increase in ROS (Fig. 2.5 and 2.6).

There was virtually no knowledge of the function of the superoxide dismutases in *D. discoideum* despite their involvement in human disease including amyotrophic lateral sclerosis (Deng et al., 1993). The current studies show that they play a role in the response of cells to xenobiotics in the environment. The antioxidant genes are downregulated by curcumin and conversely cells overexpressing SOD are more resistant to curcumin. It will be important in the future to construct a library of mutants with each of the SOD genes individually deleted so that their specific involvement in the response to xenobiotics can be accessed. Catalase A has been more extensively studied in *D. discoideum*. Both catalase A and SOD enzyme levels are down-regulated by curcumin. Previously, it was shown that its transcription was not affected by exogenous H_2O_2 (Garcia et al., 2000).

The analysis of mutants further demonstrated that protein kinase A mediates this cellular response to curcumin. The observation that the effect of curcumin on cell proliferation and down-regulation of catalase were dependent on PKA further supports the idea that curcumin does not act directly to increase ROS in the cells. PKA is a major regulator during multicellular development in *D. discoideum* and all metazoans (Loomis, 1998). Although it was known to be expressed in proliferating *D. discoideum* cells (Leichtling et al., 1984), PKA null cells divide normally (Mann and Firtel, 1991; Simon et al., 1992). Thus, this demonstration that PKA functions to regulate the response of cells to a xenobiotic suggests that this may be a normal role for the enzyme in mitotically dividing cells.

Studies in *D. discoideum* on the effects of valproic acid, a bipolar disorder treatment, have shown that the cellular response to the drugs is mediated though PKA (Boeckeler et al., 2006). This was subsequently translated and verified in mammalian cells. In addition, PKA has been shown to mediate sensitivity to the widely used anti-cancer drug cisplatin (Li, 2000; Li et al., 2000). Overall, these studies reveal a novel mechanism by which this popular herbal supplement effects cell physiology (Fig 2.26).

These findings prompted the use of RNA seq to provide an unbiased and more nuanced view of the transcriptional effects of curcumin. The results showed that there is an early and transient dose dependent effect on transcription that effects about 600 genes (approximately 5% of the genome). This is a dramatically limited
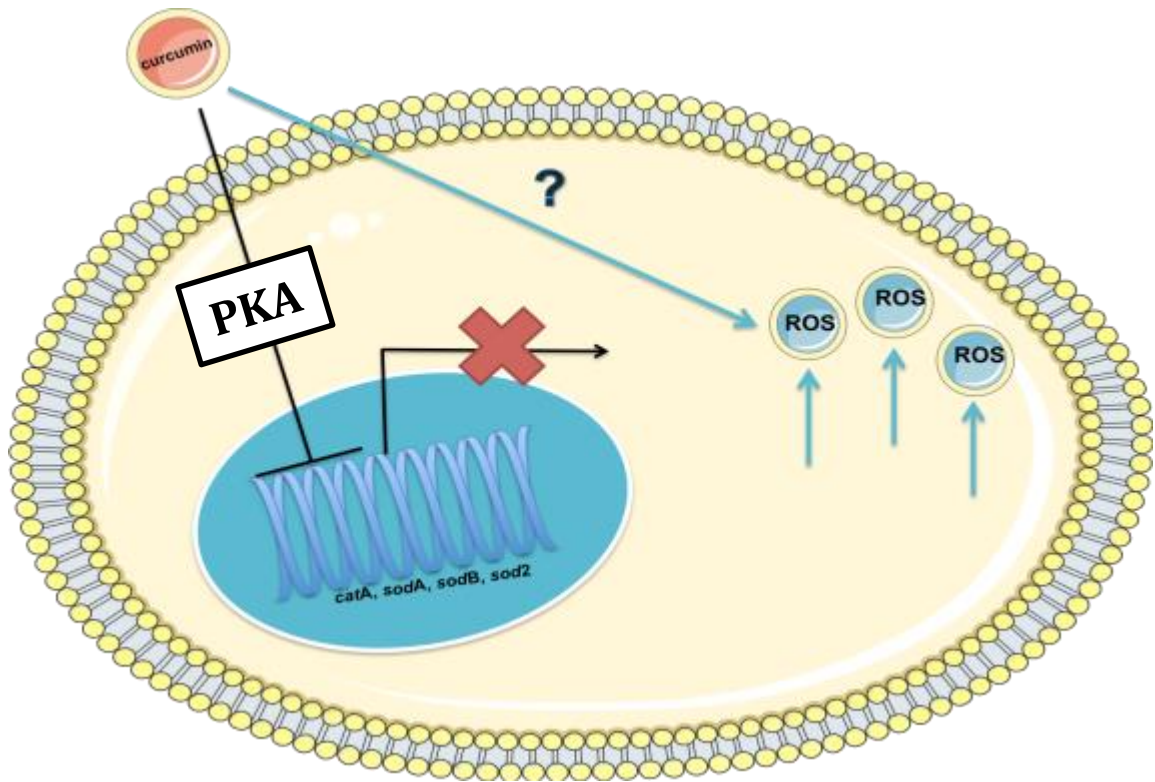
Fig. 2.26: Proposed mechanism of curcumin action. Curcumin inhibits growth and generates ROS in *D. discoideum*. Curcumin induced major changes in transcription which included the reduction of catalase A and superoxide dismutase mRNA levels through a PKA mediated pathway. The results of this study suggest the conclusion that the increase in ROS is not the cause of the decrease in antioxidant enzyme levels, but rather that the decrease in the enzymes results in the increase in ROS levels.

early response compared to the transcriptional response accompanying the transition to development in *D. discoideum* where the transcription of about one-half of the genes is affected (Parikh et al., 2010).

Curcumin quickly up-regulates the transcription of 533 genes that gene ontology analysis encode oxidoreductase enzymes, osmotic/salt/heat stress proteins, and cell cycle proteins. The early down-regulated group includes genes involved with cell death, defense to bacteria, and peroxisome function. This early transcriptional response is transient and is replaced by a subsequent change in gene expression where 204 genes are up-regulated while 443 genes are down-regulated. The later up-regulated genes encode proteins involved in oxidoreductase activity, antioxidant activity, vitamin binding and the response to abiotic stimuli. The down-regulated late genes include those involved with the cells cycle, DNA replication, and the response to drugs. The GO term enrichment lists at the early and late time points differ and further emphasize the cellular response to curcumin is multiphasic and complex. Directed biochemical analysis showed that later in the response there is the down-regulation of the antioxidant genes that is under the control of PKA. As a result, there is an increased accumulation of superoxide in the presence of curcumin.

These data on transcription and those on the effect on curcumin on cell proliferation demonstrate that curcumin seems to be working in two phases. There is an initial phase where cells die in a dose dependent manner which is accompanied by the early

transient changes in gene expression. This is followed by a durable dose dependent inhibition of the rate of cell proliferation and a silencing of the early changes in transcription.

These studies extend a previous study focused on the effect of curcumin on multicellular development of *D. discoideum* (Garige and Walters, 2015). The authors showed that curcumin inhibited both chemotaxis and multicellular development. The accumulation of the mRNAs of the early developmentally regulated genes *yak*A, *kea*A, *pka*C and *car*A was also inhibited, while two of five glutathione-S-transferase genes were upregulated. Our finding that cell proliferation of the *pka*C null and the *pka*R-overexpressing mutants is more resistant to the inhibitory effects of curcumin emphasizes the importance of this enzyme as a central regulator in the response to curcumin both in cell proliferation and development in this organism. Both studies show a pleiotrophism of effects of curcumin, but there appear to be significant differences of the effects of curcumin on proliferating and developing cells.

Further work needs to address the precise mechanism by which curcumin affects PKA and how PKA then affects transcription. Indeed, the mechanism by which the groups of early and late genes are transcriptionally controlled remains to be answered. In addition, these studies suggest new functions for genes that can be further investigated by making the appropriate null mutations.

The significant and unexpected complexity of the effect of curcumin on cell physiology revealed by these studies, underscores the possibility of negative effects and unknown drug interactions when taken therapeutically. Indeed, there is no rational dose for the human consumption of curcumin as there is no known effect or measurable endpoint.

Overall, these studies provide a new look at the effect of curcumin on cells and opens up new avenues for investigation. As in early drug studies employing *D. discoideum*, these ideas can be extended to human cells (Boeckeler et al., 2006; Min et al., 2007; Min et al., 2005b). Lastly, these results suggest the interesting idea that curcumin could be used as an amoebicide to treat diseases like amoebiasis caused by *Entamoeba histolytica*.

3. Identification of genes and molecular pathways involved in resistance to acriflavine in *Dictyostelium discoideum*

A. Abstract

Mutations conferring resistance to acriflavine in *Dictyostelium discoideum* have been assigned to four loci, *acr*A, *acr*B, *acr*C and *acr*D. However, only the gene for the *acr*A locus has been identified as the peroxisomal catalase A (*cat*A) enzyme. Mutations in the *cat*A gene also confer cross-resistance to other chemicals including methanol, but the mechanism of the cross-resistance is unknown. We have used insertional mutagenesis to isolate four putative strains with disruptions in previously uncharacterized genes which confer resistance to acriflavine. One of these mutated genes also confers cross-resistance to methanol, unlike the other three, but this is not the catalase gene. This study sought to provide more insight into acriflavine resistance, and thus provides a functional basis for genetic loci that have been widely used in *D. discoideum* genetics.

B. Introduction

Genetic differences in metabolic and signaling pathways greatly affect how an organism responds to drugs (Klotz, 2007). The use of suitable model systems in pharmacogenetic analysis allows for the subsequent characterization of genes and their protein products in mammals to explain the mechanisms of drug action. The social amoeba, *D. discoideum,* has proven to be a good genetic model for elucidating the mechanism of drug action and improving the efficacy of the drugs (Alexander et

al., 2006; Williams, 2005). The success of *D. discoideum* as a model for pharmacogenetic studies lies in the ability to identify specific genes causing drug resistance by using well-developed molecular techniques.

Acriflavinium chloride is a topical antiseptic commonly referred to as acriflavine but has also been given a variety of names depending on the manufacturer. The drug was developed in 1912 by Paul Ehrlich, who was studying how dyes could be used to visualize microbes. In addition, acriflavine was discovered to be able to treat sleeping sickness, diphtheria, syphilis and has been used as a topical antiseptic to clean minor wounds and prepare the skin for surgical procedures (Pons et al., 2001). The mechanism(s) of action of acriflavine is not known.

Acriflavine has been shown to have anti-tumor effects in mice by inhibiting the function of HIF-1, a transcription factor, which promotes angiogenesis (Lee et al., 2009). Angiogenesis, the formation of new blood vessels can be induced by tumor growth, tissue wounding and inflammation, all of which present environments of hypoxia. Mice that had Ehrlich cancer cells implanted in them and then treated with acriflavine showed a 30% inhibition in tumor growth (Kim et al., 1997). In another mouse study, acriflavine was shown to inhibit HIF-1 dimerization thus preventing tumor growth in mice with prostate cancer xenografts, and mice with tumors that were treated with acriflavine resulted in tumor arrest (Lee et al., 2009).

Acriflavine resistance has been used in *D. discoideum* as a marker for parasexual genetic studies (Williams et al., 1974). Complementation and linkage studies have demonstrated that mutations conferring resistance to acriflavine in *D. discoideum* are recessive and have been assigned to four loci, *acr*A, *acr*B, *acr*C and *acr*D (Katz and Kao, 1974; Rothman and Alexander, 1975; Williams et al., 1974). An implication of this may be that the action of acriflavine is mediated by multiple signaling pathways. Of the four loci, it was also shown that only *acr*A mutations confer cross-resistance to the unrelated compounds, methanol and thiabendazole (Williams et al., 1974). A later study identified the peroxisomal catalase A enzyme (catA) as the product encoded by the *acr*A locus (Garcia et al., 2002). Null mutants of *acr*A created by homologous recombination are resistant to both methanol and acriflavine (Garcia et al., 2002). The identity of the structural genes encoded by *acr*B, *acr*C, and *acr*D however, remain unknown.

In this study, we used the direct genetic approach of insertional mutagenesis to identify previously uncharacterized genes involved in acriflavine resistance in *D. discoideum*.

C. Materials and Methods

I. Reagents

An extinction coefficient for acriflavine was established in water (Alexander et al., 2013). A stock solution of 100 mg/ml (213.2 mM) was prepared in water and a 1:10,000 dilution in water was used to prepare an absorbance spectrum (Table 3.1).

Acriflavine
100mg/ml $\Longrightarrow$ 1:100
dilution $\Longrightarrow$ 1:100
dilution

Wavelength (nm)	Absorption
449	0.994
260	1.237
211	0.26

Table 3.1: Spectral properties of acriflavine dissolved in water. 100 mg/ml acriflavine stock was diluted $1:10^4$. Shown are the absorption peaks of acriflavine.

This was done five times and the average absorbance at 449 nm was calculated to be 1.097. Using Beer's law, the extinction coefficient was established as 1.097 μg/ml/cm. Thus, we routinely made new stock solutions at approximately 500x the required final concentration, read the absorbance and adjusted the final concentration to exactly 1x based on the absorbance reading. Clearly, this approach is dependent on the absolute concentration of our initial stock solution, but it allows us to always prepare the stock solution and plates precisely the same way.

II. Strains and conditions for growth

Parental strain Ax4 was used for all experiments including insertional mutagenesis and maintained as described in Chapter 2. Previous reports of acriflavine resistance in *D. discoideum* used 100 μg/ml as the concentration for mutant selection and testing (Williams et al., 1974). These studies were all done with the parental strain NC4, which is not capable of growth in axenic HL-5 medium. Since REMI is restricted to the use of strains that were selected for growth in axenic medium, we tested the sensitivity of axenically grown Ax4 on plates containing different concentrations of acriflavine. 10^6 or 10^2 cells were plated in duplicate on plates containing different concentrations of acriflavine and on SM plates lacking acriflavine. These two cell concentrations were used to show that there is a discrete acriflavine concentration where neither of the innocula would proliferate. This ruled out the possibility that a high cell number could provide some type of resistance and effectively preclude the isolation of rare mutant cells. Parallel plating the cells on SM plates allowed the direct determination of the number of live cells in the innocula. We observed that at

acriflavine concentrations below 200 µg/ml, the 10^6 cell inoculum cleared the plates of the bacterial food source after two days indicating that there was insufficient growth inhibition to allow for mutant selection. We observed the same pattern with the 10^2 inoculum except that the surviving colonies could be counted and the results presented quantitatively (Fig 3.1). The data show a dose dependent inhibition of cell growth as assessed by colony formation, and established 200 µg/ml as optimal for selection of resistant mutants.

III. Restriction enzyme mediated integration (REMI)

Five independent transformations were performed, each with a cell density of 1.5×10^7 cells. Cells were electroporated with *DpnII* restriction enzyme along with *BamHI* linearized pBSR1 plasmid, which contains the gene for blasticidin S resistance (BSR) (Shaulsky et al., 1996), to facilitate integration of the plasmid into the host genome. *DpnII* binds the recognition sequence 5'-GATC-3' and cleaves these sequences just before the 5'-G on each strand. *BamHI* linearized pBSR1 plasmid has a four base 5'-GATC-3' overhang which makes it compatible with the *DpnII* site of integration (Fig 3.2).

Transformants were selected for growth in HL-5 medium containing 10 µg/ml blasticidin S. These transformants were subsequently plated on plates containing 200 µg/ml acriflavine. In each case there were colonies that displayed robust growth in the presence of acriflavine. One clone from each transformation (P1-P5) was picked and retested for their ability to grow in the presence of 200 µg/ml acriflavine.
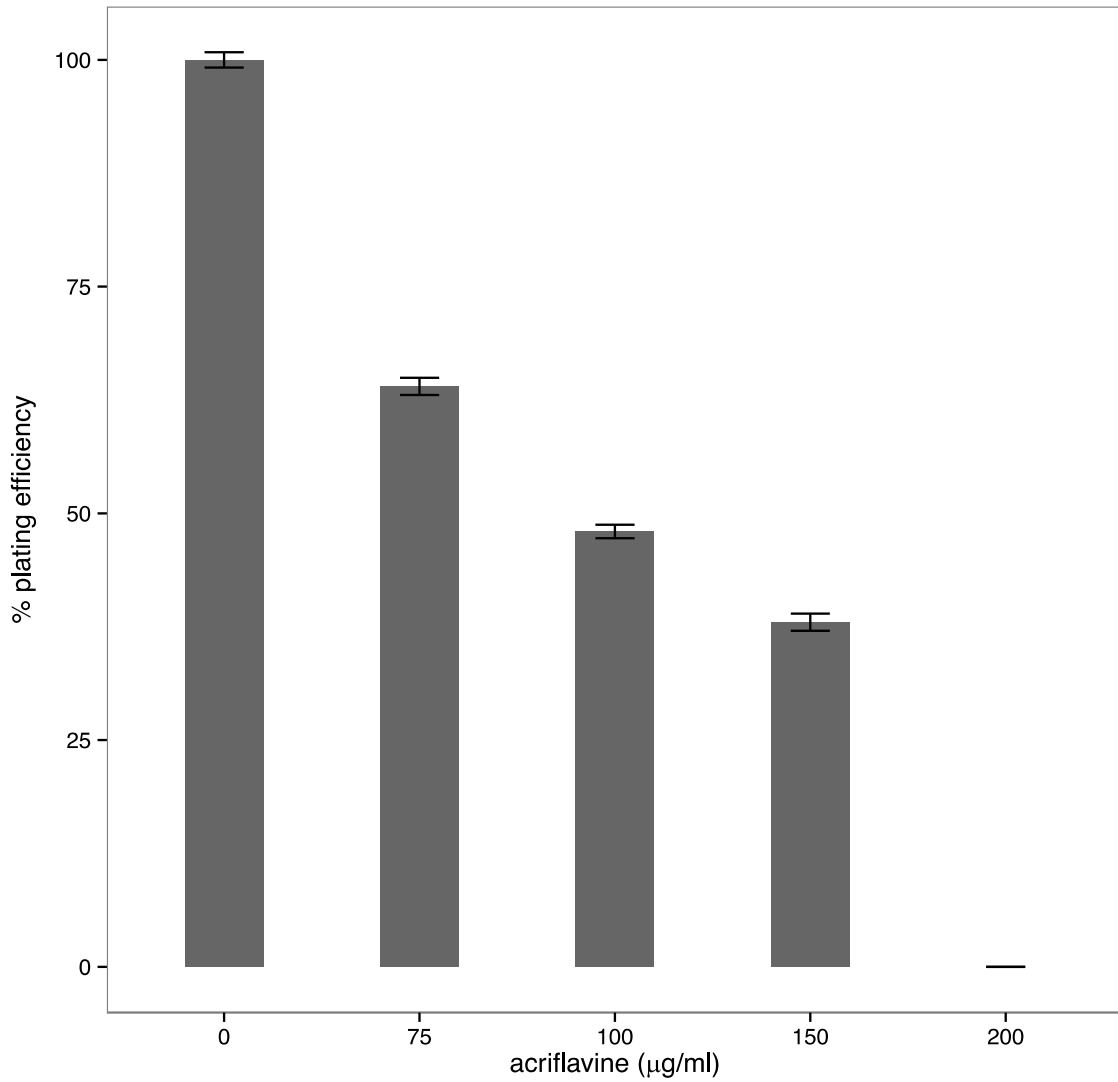
Fig. 3.1: Determination of working concentration of acriflavine in SM agar plates. Parental Ax4 cells were serially diluted and plated in association with *K. aerogenes* on SM agar plates containing increasing concentrations of acriflavine. Complete inhibition of cells is only observed at 200 μg/ml.
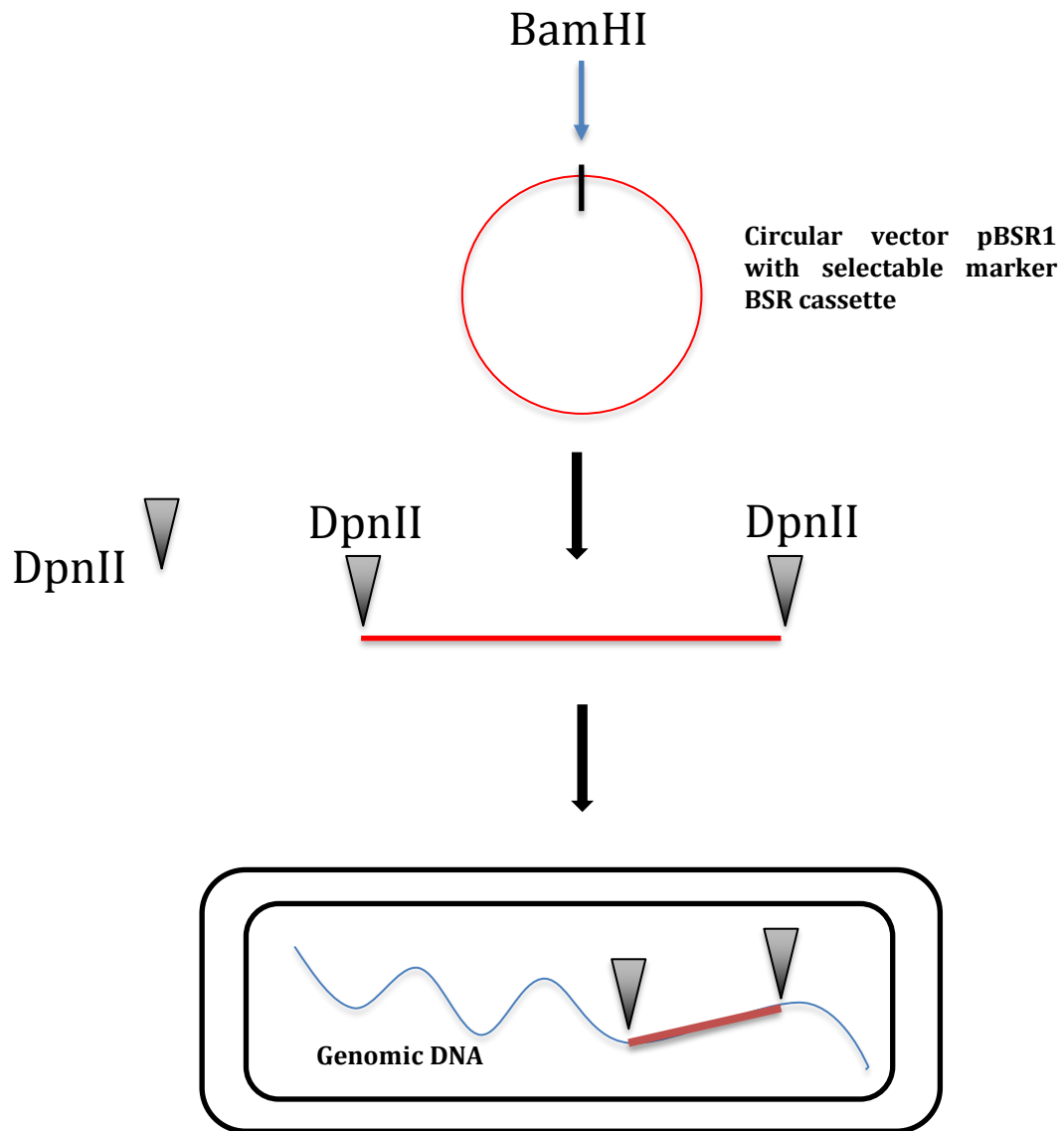
Fig. 3.2: Restriction enzyme mediated integration (REMI). pUCBsrΔBam plasmid with BSR cassette (pBSR) is randomly inserted into genomic DNA. Blasticidin resistant transformants are tested for growth on 200 µg/ml acriflavine. An insertion in the correct gene will result in resistance to acriflavine.

IV. Cell Survival Assay

Transformants of each strain were serially diluted and plated in association with *Klebsiella aerogenes* onto SM agar plates containing 200 µg/ml acriflavine or 2% methanol. As each cell grows, they ingest the bacteria and subsequently form a plaque that can be counted.

V. Inverse PCR or Plasmid Rescue

To identify the mutated genes in the insertional mutants, genomic fragments flanking either side of the disruption vector used in the transformation were sequenced based on an inverse PCR technique (Keim et al., 2004). This technique takes advantage of the knowledge of the sequence of the inserted plasmid and the average fragment length of 1050 bp when the genomic DNA is digested with *AluI*. *AluI* cuts within the coding region of the BSR cassette, which is close to the point of insertion (Fig 3.3). Between the point of insertion and the BSR gene, however, there is no *AluI* site. Using primers BSR 339 (5'-GATGCTACACAATTAGGC-3') and BSR 347 (5'-ATGCCGCATAGTTAAGCCAG-3') (see table 3.2 for list of primers) the genomic fragment flanking the insertion generally can be identified by sequencing.

In the event that we could not identify the disrupted gene by inverse PCR, as was the case of putative mutant strains, P1 and P5, we digested genomic DNA from transformants with *EcoR1* (found in the genomic sequence but not the REMI vector).
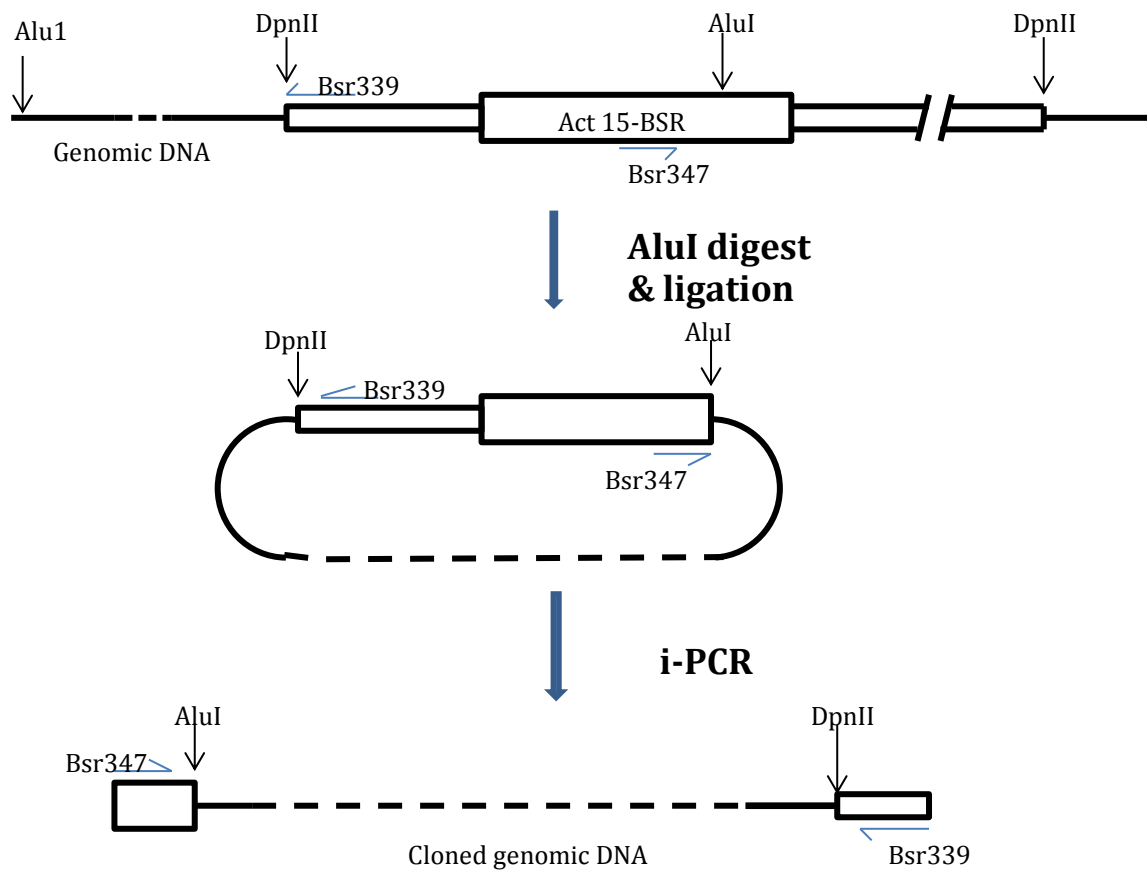
Fig. 3.3: Inverse PCR. REMI-mutant transformants are digested with the restriction enzyme *Alu*1 and ligated with T₄ DNA ligase. An inverse PCR reaction is set up using primers that anneal to the Bsr gene. Adapted from Keim et al. 2004.

Primer ID	Primer Sequence
178	5' CGT ACG AAT TGC CGC TCC C 3'
255	5' GGG AGC GGC AAT TCG TAC G 3'
339	5' GAT GCT ACA CAA TTA GGC 3'
347	5' ATG CCG CAT AGT TAA GCC AG 3'
387	5' CGC GGA TTCC GCG TAT TTC CAC CAC CAA TTG 3'
397	5' GGC AAG CAT TTT AGG AGA 3'
466	5' GAG AGT CGA CAT TTA ACA ACT ACA GTA GGT TT 3'
467	5' GAG AGG ATC CGT GGT GAT TGT GTT GAA GTT 3'
468	5' GAG AGG ATC CAT TGT AAT TAT TAT TGT TTA T 3'
469	5' GAG AAG ATC TAT AAA AAA AAT TAT TCA CTA ACA ACA 3'

Table 3.2: List of primers used in acriflavine study.

In this way we could rescue the plasmid and clone the genomic sequence flanking the insertion into DH5α *E. coli* (Fig 3.4).

The sequences generated from these two techniques were checked against the genome sequence using the BLAST algorithm program at Dictybase, an online bioinformatics database for the model organism *D. discoideum*. Gene information is summarized in Table 3.3.

VI. Catalase Activity Assays

5×10^6 cells of the putative acriflavine resistant mutants and parental Ax4 cells were lysed in 100 µl lysis buffer (10 mM potassium phosphate, pH 7.0, 0.1% Triton X-100, 1X protease inhibitor). 5 µl samples were assayed for catalase activity by mixing with 1 ml 10 mM H_2O_2 in 50 mM potassium phosphate, pH 7.0 and the breakdown of H_2O_2 is monitored over a two-minute period at 10 second intervals with a spectrophotometer. Optical density is used as a measure of peroxide concentration since optical density increases linearly with peroxide concentration. The absorbance of H_2O_2 is measured at 240 nm since its products, water and oxygen, do not absorb at this wavelength.

VII. Recapitulation of mutants by homologous recombination

To confirm that the observed phenotype, acriflavine resistance, was a result of a single disruption in the identified genes and not a secondary mutational event, a
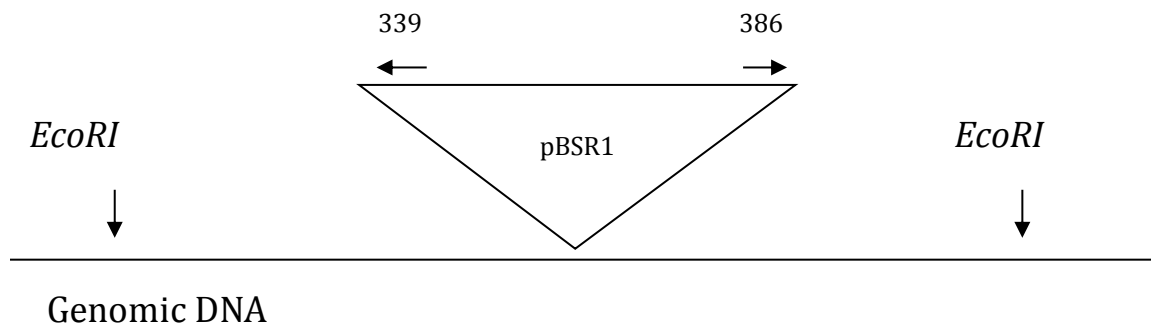
Fig. 3.4: Cloning by restriction digest. Genomic DNA is digested with *EcoRI* (not found in plasmid) and cloned into DH5α. Purified recombinant DNA is re-digested and sequenced with primers that anneal to pBSR1.

Putative Mutant Strain	Method used to determine gene	Chromosome No: Position	Gene ID	Protein size
P1	i-PCR / cloning by restriction enzyme		Not identified	
P2	i-PCR	2: 6897934-6902781	DDB0307594_G02276559	1615aa
P3	i-PCR	4:4843651-4843802	DDB0306460_G028661	813aa
P4	Cloning by restriction enzyme	2:3173987-3174629	DDB0348514_G0273621	454aa
P5	i-PCR	2:6897934-6902781	DDB0307594_G02276559	1615aa

Table 3.3: Gene information for identified disruptions.

disruption vector was generated for homologous recombination to recapitulate the mutation.

<u>DDB0307594 disruption vector</u> (Fig 3.5A). A 1083 base pair (bp) *NdeI-ClaI* fragment based on sequence analysis from the 3' end of DDB0307594 was generated by PCR using primers that specifically annealed to the gene (Table 3.2). Ax4 genomic DNA was used as a template. The *NdeI-ClaI* amplicon was cloned into *NdeI-ClaI* digested pSP72, a cloning vector from Promega to generate pSPC7. There is a *BamHI* restriction site 542 bp downstream from *NdeI* on the amplicon. A 1400 bp *BamHI* fragment, containing the BSR cassette from plasmid, SL63 (available from www.dictybase.org), was cloned into this *BamHI* site of pSPC7 to generate pSPC7BSR. To confirm the nucleotide sequence in the vector, we sequenced the *NdeI-ClaI* fragment of DDB0307594 disruption vector with primers that flank the respective restriction sites.

<u>DDB0306460 disruption vector</u> (Fig 3.5B) Based on the nucleotide sequence of DDB030640, two fragments of DNA homologous to the 3' end of the gene were amplified using Ax4 genomic DNA as template (Table 3.2). These fragments, approximately 500bp each were cloned separately into cloning vector, pSP72. The *bsr* gene, a 1400bp *BamHI* fragment from SL63 vector containing the resistant marker, was cloned into the resultant vector bearing the two arms homologous to DDB0306460. The genomic fragment to be amplified has no cloneable sites and so *SalI*, *BamHI* and *BglII* restriction sites were engineered into the sequence, as this
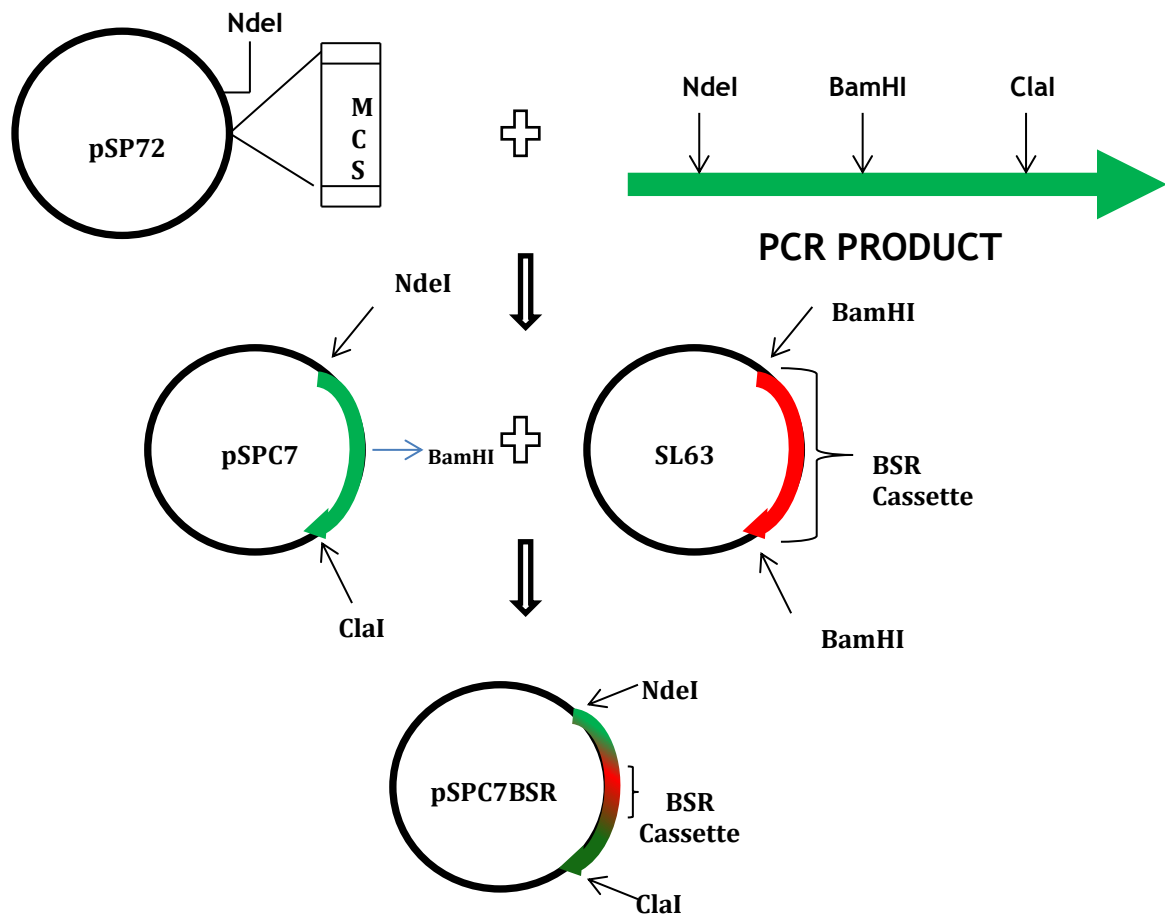
Fig. 3.5A: Construction of disruption vector pSPC7BSR. An *NdeI-ClaI* fragment is amplified using Ax4 genomic DNA as template and cloned directly into corresponding sites of pSP72 resulting in pSPC7. A BSR cassette flanked by *BamHI* restriction sites is then cloned into the *BamHI* site of pSPC7 resulting in the disruption vector, pSPC7BSR.
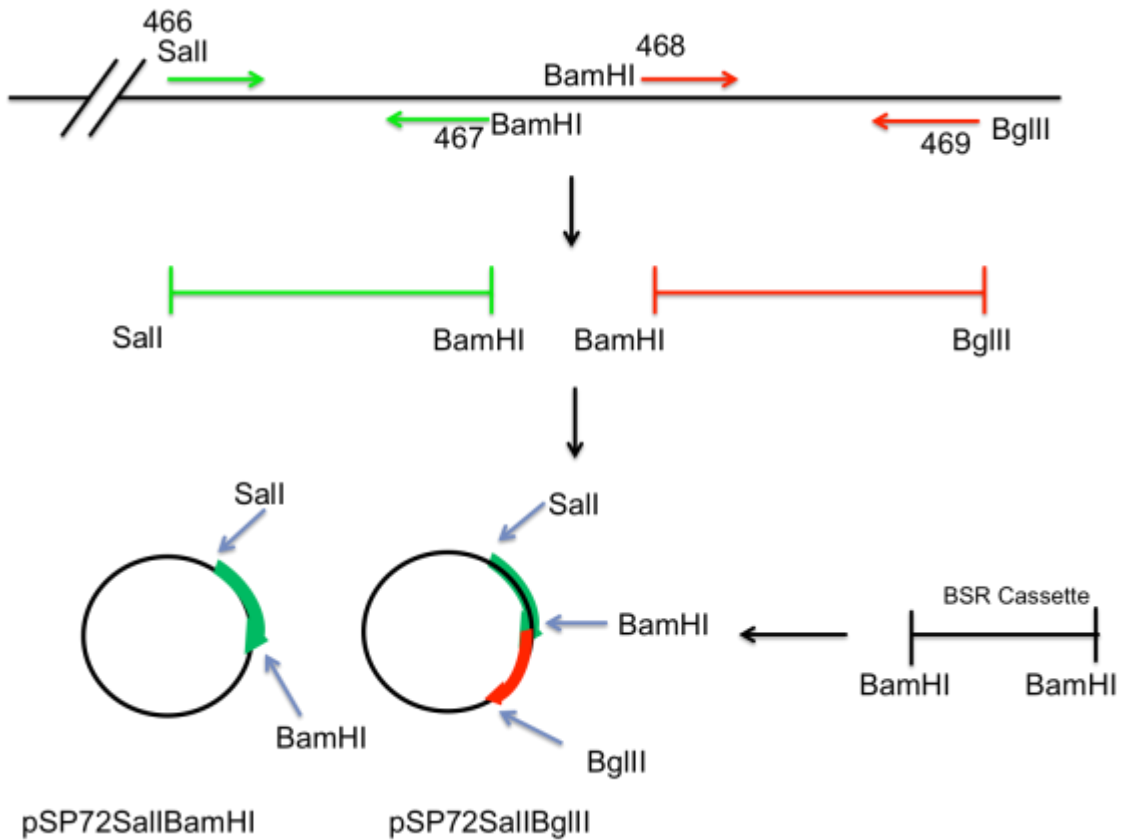
Fig 3.5B Construction of disruption vector pSPC26460*SalIBglII*. A *SalI-BamHI* fragment will be cloned directly into corresponding sites in pSP72 resulting in pSP72*SalIBamHI*. The *BamHI-BglII* fragment will then be directly cloned into pSP72*SalIBamHI* resulting in pSP72*SalIBglII*. The 1.4kb BSR cassette from SL63 will be cloned into the *BamHI* site of pSP72*SalIBglII*.

would have been necessary for cloning. To ensure precision of nucleotide sequence in the vector, we sequenced the 2.4 kb *SalI-BglII* fragment of DDB0306460 disruption vector with primers that flanked the respective restriction sites and referred to the disruption vector as pSPC26460*SalIBglII*.

DNA and transformation by electroporation was performed based on a protocol used for DNA-mediated transformation (Gaudet et al., 2008). In order to increase transformation efficiency, HL-5 medium was supplemented with 0.2 g/ml folic acid and 0.6 g/ml vitamin B12 for two weeks before transformation. 1.5×10^7 Ax4 cells were transformed with 20 μg excised DNA fragment at 1 kV and 3 μF in 4 mm-gap width cuvettes using a Bio-rad gene pulser. Transformed cells were transferred to 10 mm dishes containing a final volume of 10mL HL-5 and incubated at 22°C. 10 μg/ml blasticidin was added to plates 24 hours later.

VIII. Confirmation of pBSRI insertion in P2 REMI

To confirm insertion of plasmid in putative strain P2, primers designed to align with genomic DNA (387 and 397) and plasmid DNA (178 and 255) were used to amplify regions that would confirm both insertion and orientation of inserted plasmid.

D. Results

I. Acriflavine resistant transformants (REMI).

Electroporation using the REMI technique (Kuspa and Loomis, 1992) generated a population of cells, each containing a single random insertion with the *bsr* gene. After selection in blasticidin, surviving cells at a density of 1×10^6 were plated in association with *K. aerogenes* on SM agar plates containing 200 µg/ml acriflavine. Transformants were allowed to give rise to single plaques and a total of 24 clones were generated from all five transformations (Fig 3.6). Subsequently, one clone from each transformation was picked, serially diluted and retested for acriflavine resistance on SM agar plates containing acriflavine. Acriflavine resistance was confirmed by establishing the efficiency of plating (EOP) on agar containing acriflavine in comparison to agar lacking acriflavine. Fig 3.7 shows that all 5 strains had a high (> 80%) EOP on acriflavine compared to the parental Ax4 strain that is not acriflavine resistant.

III. The acriflavine resistance is not due to disruption of the *acr*A (*cat*A) gene

Previous genetic studies showed that all *acr*A mutations selected on acriflavine are also resistant to methanol. Conversely, all mutants were selected directly on methanol are also resistant to acriflavine and do not complement *acr*A mutations. A detailed molecular study of methanol resistance using insertional mutagenesis demonstrated that the affected gene was the catalase A gene. Further study of archived methanol resistant and *acr*A mutants showed that each of them lacked
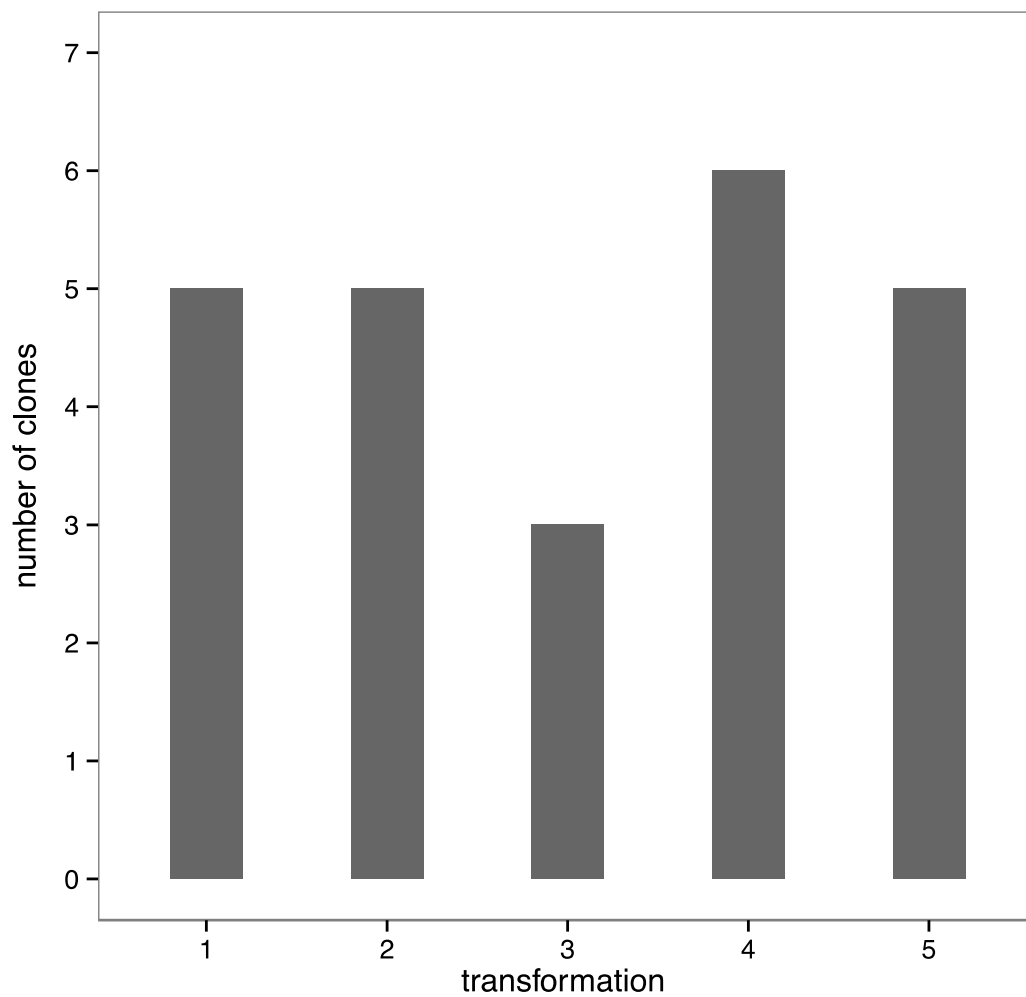
Fig 3.6: Acriflavine resistant clones generated from REMI. Acriflavine resistant clones were generated from each of the five transformations.
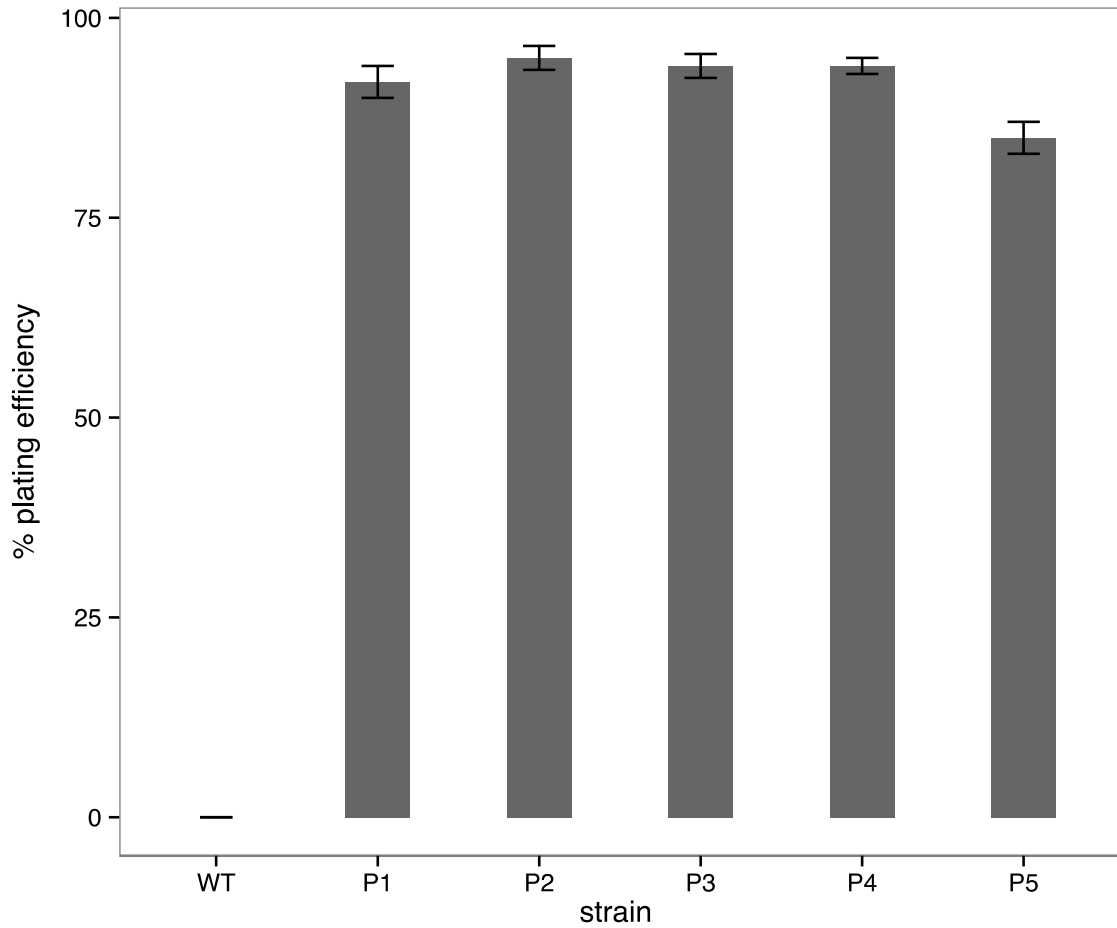
Fig. 3.7: Acriflavine resistant mutants from REMI. Plating efficiency showed that each acriflavine resistant mutant generated and selected from each REMI had over 80% efficiency of plating compared to the parental Ax4 strain.

catalase A enzyme activity, by a variety of mechanisms including lack of mRNA expression and deletion or mutation of the *cat*A gene (Garcia et al., 2002). Thus, the lack of catalase A enzyme activity is diagnostic for mutations at the *acr*A locus.

The P1-P5 strains were assayed for catalase A enzyme activity and these results are shown in Fig 3.8. The data demonstrate that mutants P1, P2, P3 and P5 all have catalase A levels equal to that of the parental Ax4 cells. Therefore, these strains express normal levels of active catalase A. In contrast, P4 lacks catalase A enzyme activity, suggesting that the *cat*A (*acrA* gene) is not functional.

IV. Acriflavine and methanol resistance

The data in Fig 3.8 suggested that only strain P4 will have cross-resistance to methanol. This idea was directly tested by plating the strains on plates containing 2% methanol along with a parallel re-examination of acriflavine resistance (Fig 3.9). As predicted all the strains were resistant to acriflavine but only P4 was resistant to methanol as well. The parental strain Ax4 was sensitive to both acriflavine and methanol. These analyses of catalase activity and methanol resistance together support the idea that strains P1, P2, P3 and P5 contained mutations in loci other than *acr*A.
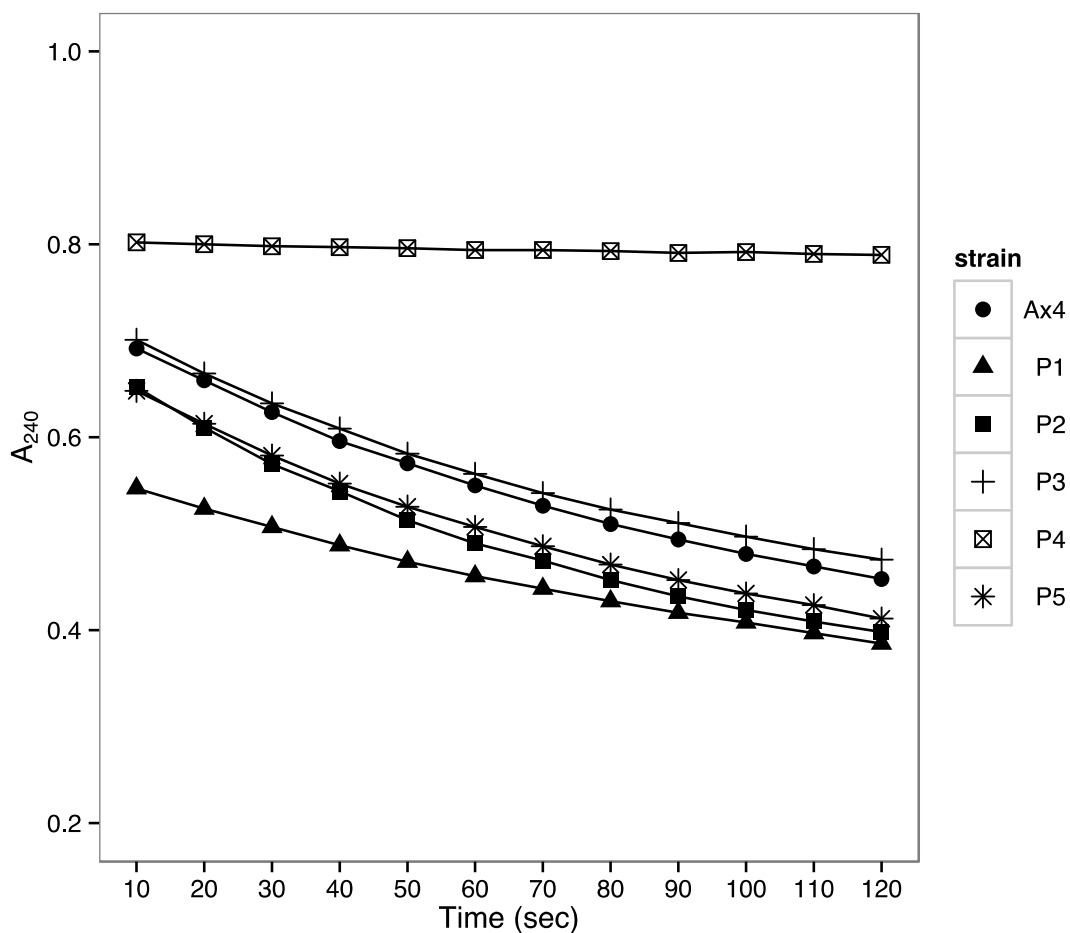
Fig 3.8: Spectrophotometric catalase activity of five acriflavine mutant strains and parental Ax4. Earlier studies showed all *acr*A mutations resistant to acriflavine were catalase deficient. As such, putative acriflavine resistant strains generated from REMI were tested for catalase activity. All strains demonstrated catalase activity by degrading H_20_2 over a two-minute time period with the exception of putative strain P4.
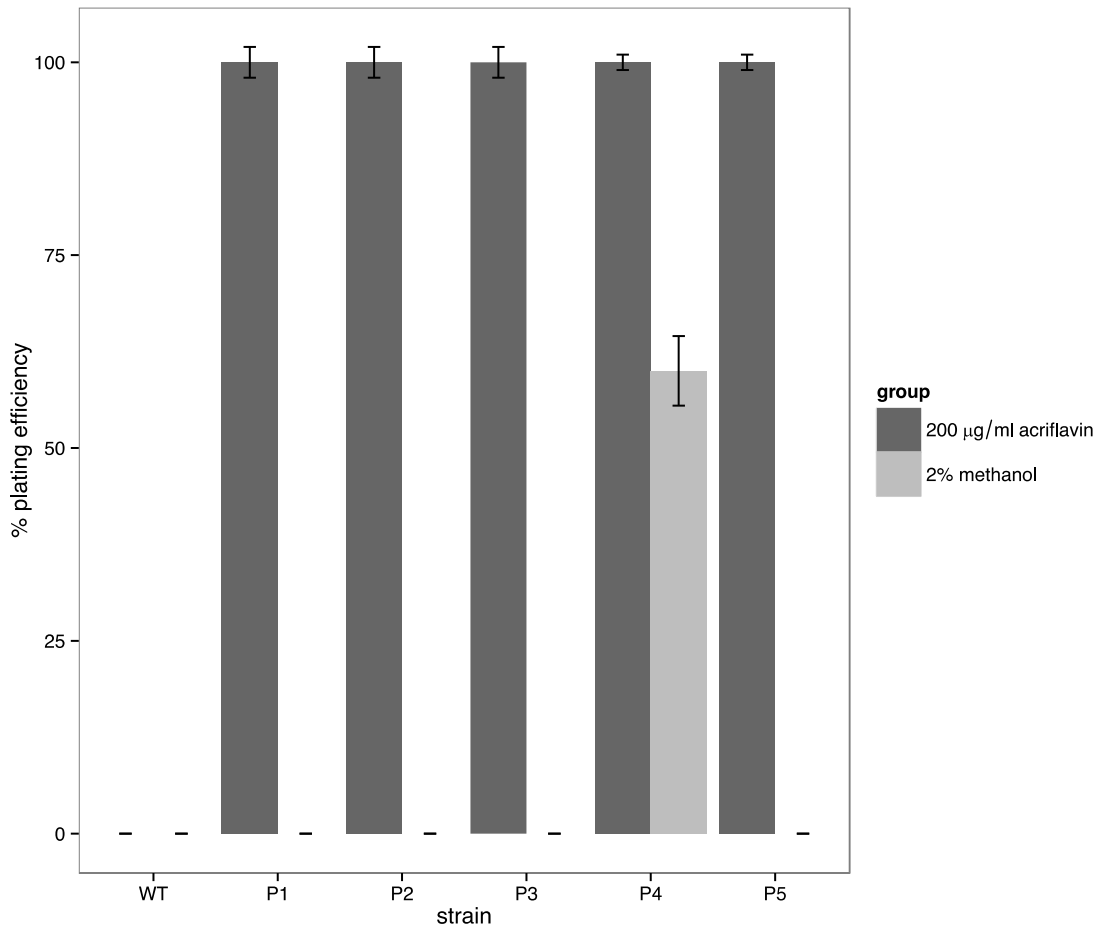
Fig 3.9: **Both wild type and mutant strains grow on agar plates minus acriflavine**. Wild type Ax4 is sensitive to 200 μg/ml acriflavine and 2% methanol and fails to grow. The five mutant strains, however, are resistant to acriflavine and 2% methanol except P4 which is resistant to 2% methanol. Plating efficiency is the ratio of colony forming cells on plates with drug to plates without drug expressed as a percentage.

V. Identification and insertion sites of genes associated with acriflavine resistance

To isolate the genomic fragments flanking the disruption vector in each acriflavine resistant transformant, one of two techniques was employed. Table 3.3 shows the method used to identify the disrupted gene in each of the putative acriflavine resistant mutants, their location in the genome, gene ID and putative protein product size. Sequencing of the genomic flanking regions of inserted plasmid identified insertions in 4 of the 5 putative mutants (P2-P5). Table 3.3 shows that the same gene was disrupted in two independent transformations (P2 and P5).

Strain P1: Identification of the disrupted gene in strain P1 could not be determined either by inverse PCR or cloning be restriction digest.

Strain P2: The identified gene was DDB0307594_G02276559. This uncharacterized gene codes for a protein that is 1615 amino acids long and 192 kDa. Blast search with amino acid sequence or nucleotide sequence did not yield any orthologs.

Strain P3: The identified gene was DDB0306460_G028661. This uncharacterized gene codes for a protein that is 813 amino acids long and 91 kDa. Blast search with amino acid sequence or nucleotide sequence does not yield any orthologs.

Strain P4: Disruption of an unknown gene in strain P4 conferred methanol resistance. The only gene that had been shown to be associated with methanol resistance in *D.*

discoideum was *cat*A (Garcia et al., 2002). This study demonstrated that cells with catalase deficiency displayed increased resistance to methanol. Although strain P4 was deficient in catalase activity, cloning by restriction digest, however, identified DDB0348514_G0273621 as the disrupted gene and not *cat*A. Blast search with amino acid sequence or nucleotide sequence does not yield any orthologs.

Strain P5: The gene identified to have a BSR insertion in strain P5 was DDB0307594_G02276559. This was the same gene identified for strain P2 generated through an independent transformation different from that which generated P5.

VI. Construction of knockout vectors for gene disruption by homologous recombination

To recapitulate the mutations and show that resistance to acriflavine was a result of a single insertion event, we attempted to disrupt the genes by homologous recombination.   The   genes   were   DDB0307594_G02276559   and DDB0306460_G028661. We chose to disrupt DDB0307594_G02276559 first because this gene had been identified from two independent transformations to confer increased resistance to acriflavine. DDB0306460_G028661 was the second gene we attempted to disrupt. We were not able to start construction of a disruption vector for DDB0348514_G0273621.

DDB0307594 disruption vector: The 1083 bp *NdeI-ClaI* genomic fragment which would serve as the 5' and 3' arms for subsequent homologous recombination was

successfully cloned into cloning vector pSP72 (Fig 3.10) and named pSPC7. The resulting vector was digested with *Cla*I to confirm insertion (Fig 3.11A). Figure 3.11B shows the 1400 bp BSR gene from vector SL63 that was cloned into pSPC7. Successful cloning of the BSR gene into pSPC7 produced an expected 4800 bp vector that included the BSR gene flanked by approximately 500 bp on each side (Fig 3.11C). Restriction digest with *ClaI* produced a single 4800 bp fragment and restriction digest with *BamHI* dropped the 1400 bp BSR cassette confirming successful insertion of BSR gene into pSPC7 which we called pSPC7BSR (Fig 3.11D).

DDB0306460 disruption vector: The 520 5' *Sal*I/*Bam*HI arm and 420 3' *Bam*HI/*Bgl*lI arm were amplified (Fig 3.12A). The 5' arm was successfully cloned into pSP72 (Fig 3.12B). Restriction digests of clone 2 with *SalI* and *BamHI* produced a single band of 2970bp and a restriction digest with *XbaI* did not produce a band of the same size confirming successful cloning on the 5' arm for the knockout vector. The vector at this point with the 5'arm cloned into pSP72 was pSpC26460*SalI/BamHI*. The 3' arm was also subsequently cloned into the resulting vector. Restriction digests with *BamHI* (Fig3.12C) and sequencing (not shown) confirmed this. After many attempts using several strategies, I was never able to finally insert the BSR cassette into pSPC2640*SalI/BamHI* to construct the final disruption vector.
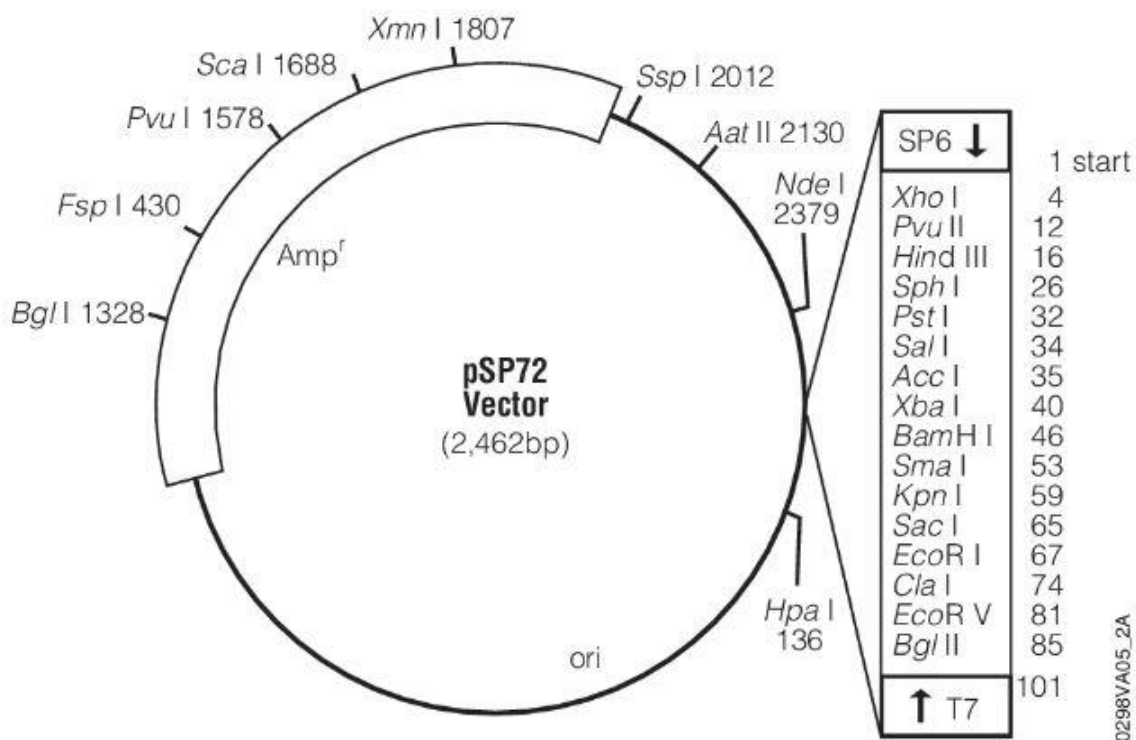
Fig 3.10: Vector sequence of cloning vector pSP72 (www.promega.com)
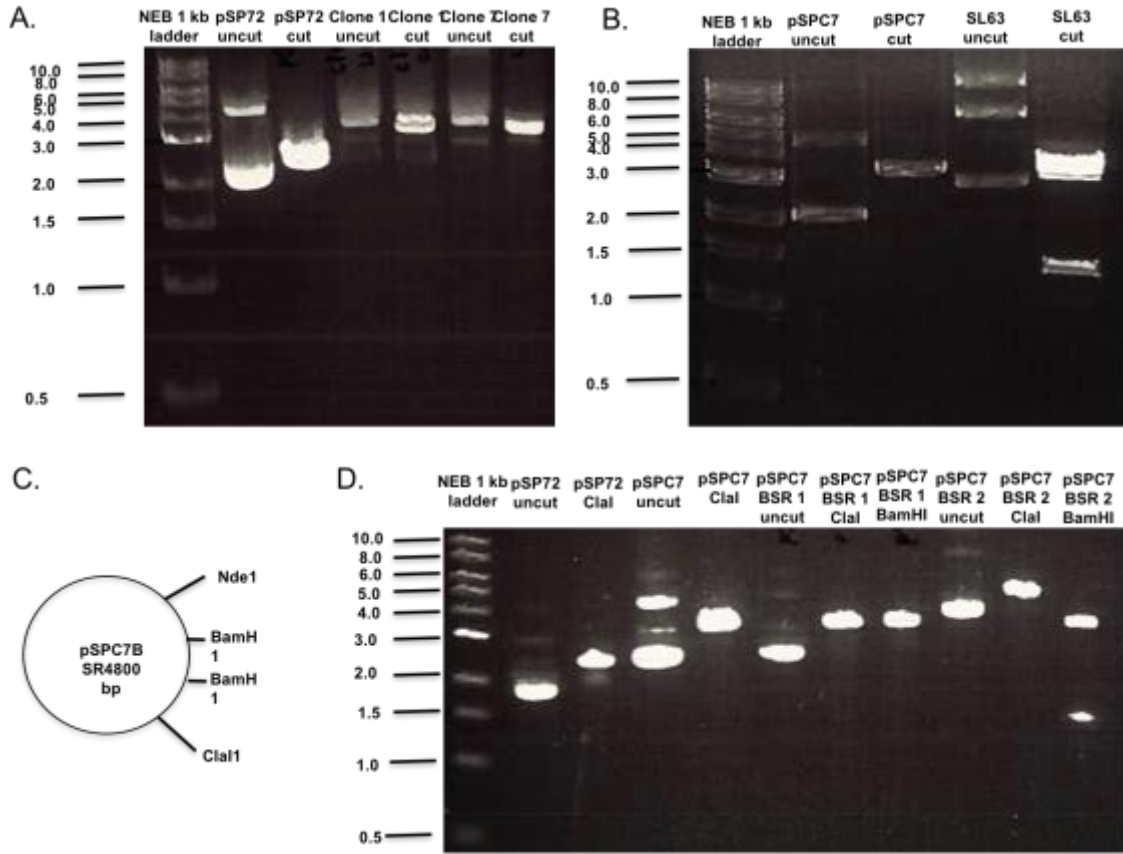
Fig 3.11: Confirmation of construction of DDB0307594 disruption vector (pSPC7BSR) A) Restriction digest of cloning vector pSP72 with *ClaI* produces the expected 2462 bp fragment. Restriction digest of both clones with *BamHI* confirms insertion of the 1083 bp genomic fragment inserted into pSP72 by producing a 3388 fragment. B) DNA gel shows the 1400 bp BSR fragment after BamHI digestion that will be cloned into pSPC7. C) Vector produced after insertion of BSR gene into pSPC7. D) Restriction digest of clone 2 with *Cla*I produces the expected 4800 bp fragment while restriction digest with *BamHI* drops the 1400 BSR cassette.
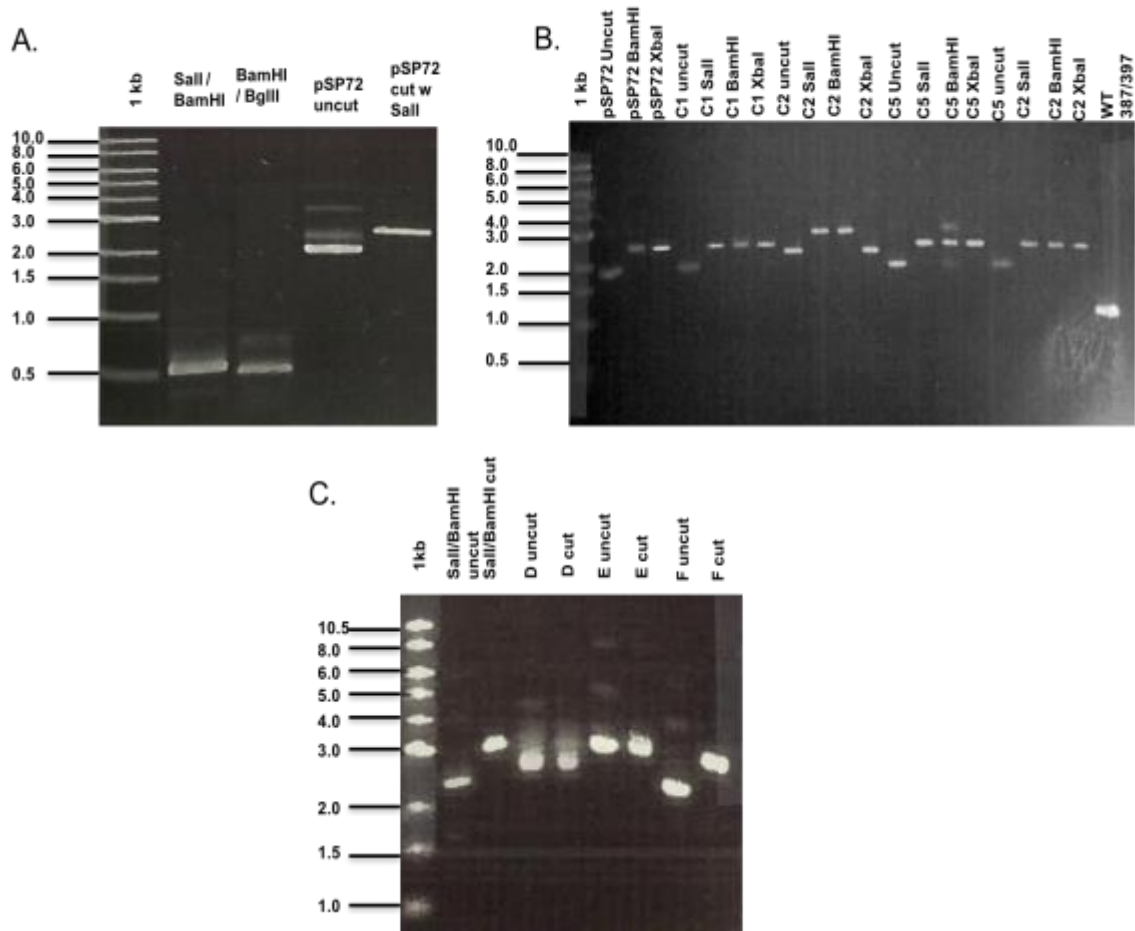
Fig 3.12: Construction of DDB0306460 disruption vector (pSPC7BSR). A) The 520 bp 5' arm and 480 bp 3' arm are successfully amplified. B) The 5' arm is cloned into vector pSP72 and produces a 3kb vector, pSPC2*SalIBamHI*. This is confirmed by restriction digest with either *SalI* or *BamHI*. C) The 3' arm is successfully cloned into pSPC2*SalIBamHI* and is confirmed by restriction digest with BamHI and sequencing analysis (not shown).

VII. Recapitulation of mutants by homologous recombination

Several attempts at recapitulating the mutation in putative strain P2 (DDB0307594) were not successful. Clones with insertions displaying BSR resistance were selected, but the insertion was never in the homologous target gene. (Fig 3.13).

To confirm original disruption in putative strain P2 which conferred resistance to acriflavine was in gene DDB0307594, PCR analysis was performed (Fig 3.14).
PCR analysis using primers that aligned with genomic and plasmid DNA confirmed that pBSR1 plasmid was inserted in gene DDB0307594 of putative strain P2 (Fig 3.15).

E. Discussion

Genes responsible for acriflavine resistance have been identified in fungi and bacteria. Molecular cloning of most of the genes identified multiple drug resistance (MDR) pumps that transport acriflavine out of the cell (Masaoka et al., 2000; Nakaune et al., 2002; Pereira et al., 1998). MDR is one of the most common mechanisms of decreasing intracellular drug concentration. By using energy derived from ATP hydrolysis, these proteins are able to move molecules across cellular membranes against a concentration gradient.
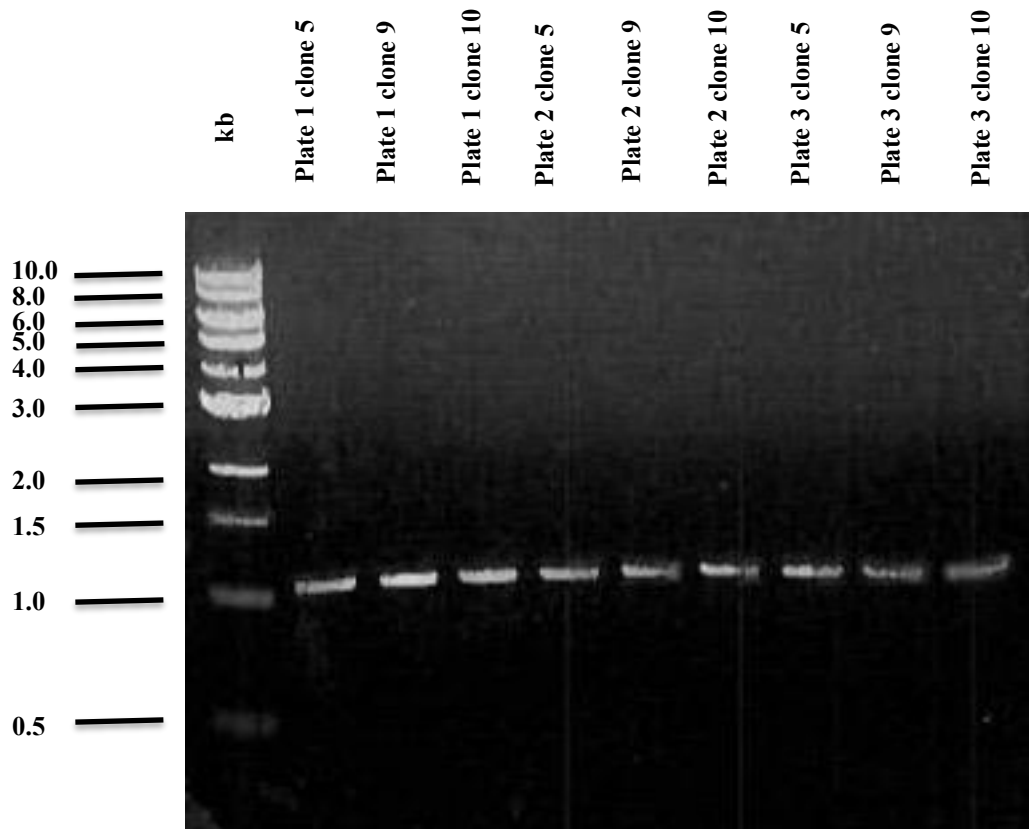
Fig 3.13: Recapitulation of REMI P2 mutation. PCR analysis of parental Ax4 cells that were electroporated with knock out vector pSPC7BSR to delete DDB0307594 did not confirm successful insertion of vector in the right gene. Correct insertion in DDB0307594 should have produced a 2480 bp fragment as opposed to the 1083 fragment expected for the parental strain.
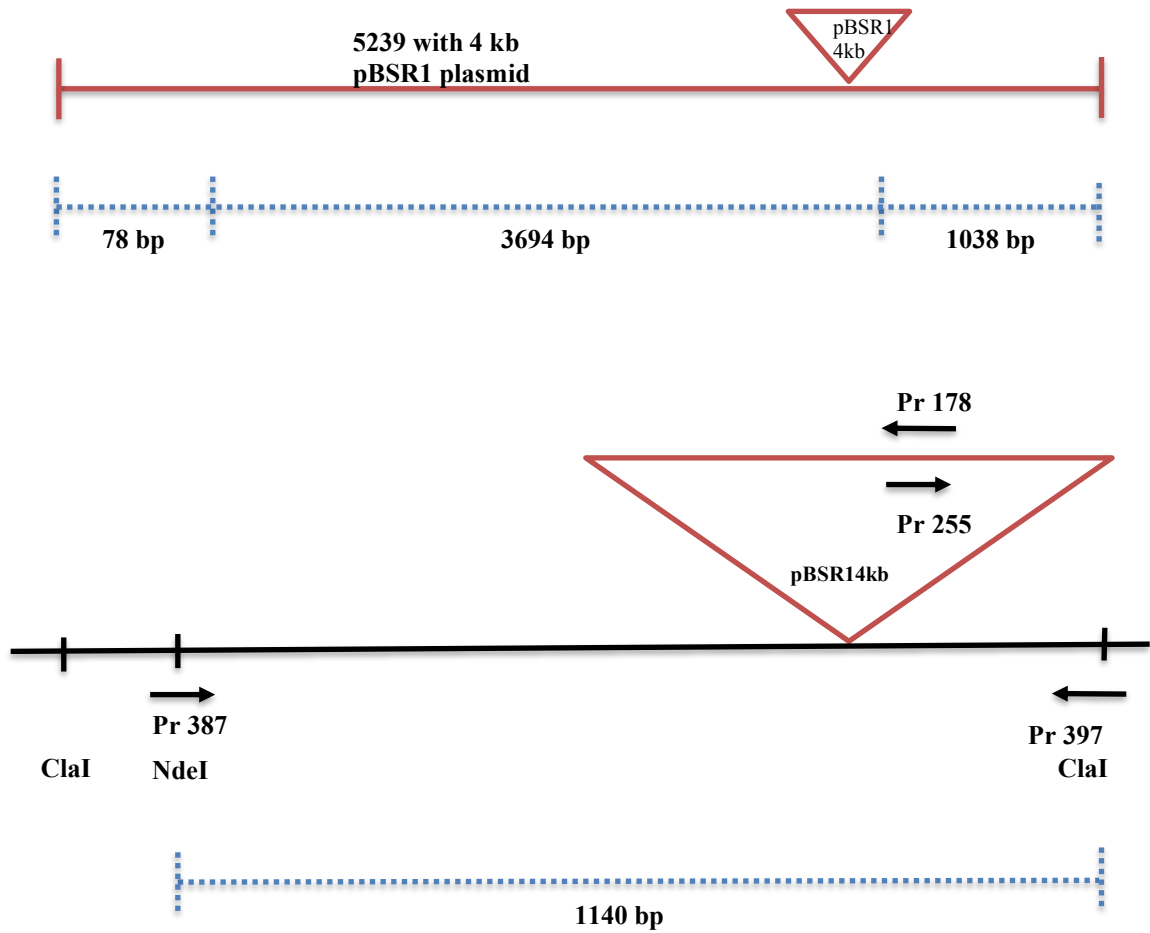
Fig 3.14: Experimental design confirming P2 REMI insertion. Primers 387 and 397 are homologous to the genomic DNA. Primers 178 and 255 are homologous to plasmid DNA and will be used to confirm insertion and orientation of plasmid.
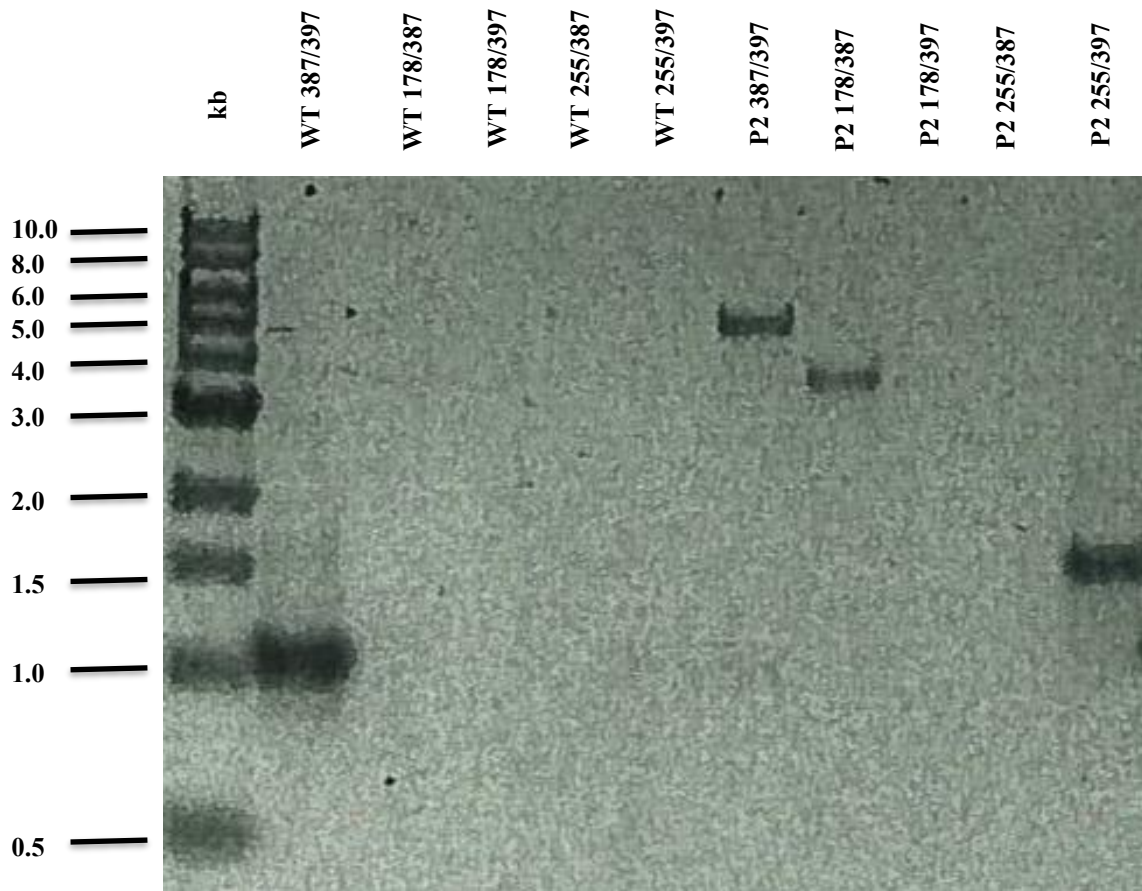
Fig 3.15: Confirmation of insertion of pBSR1 in P2. A single 5272bp band produced by primers 387 and 397 the acriflavine resistant P2 strain confirms insertion of plasmid. Compare to the 1083 band produced in the parental Ax4 strain (wild type) that does not have an insertion. The band produced using primers 178 and 387 also confirmed orientation of plasmid.

MDR has been studied only superficially in *D. discoideum* (Anjard et al., 2002; Brazill et al., 2001)*.* There are seventy-one ABC transporters in *D. discoideum* belonging to six different families; A, B, C, D and G. Comparisons have been made to animal, plant, and fungal orthologs in order to gain some insight into the evolution of this superfamily (Anjard et al., 2002). It is possible that acriflavine resistance in *D. discoideum* may be due to transport across the cell membrane since a prerequisite for the efficacy of many drugs is the ability to traverse the cell membrane and interact with their targets. However, none of the newly identified acriflavine genes was a member of this gene family.

We identified three of the four genes whose disruption conferred acriflavine resistance. (P2/P5) DDB0307594, (P3) DDB0306460 and (P4) DDB0348514 are all uncharacterized genes with no known functions. Recapitulation of the mutations from the random mutagenesis would have allowed us to assign a biological role for these genes. These genes could not have been essential as transformants from random mutagenesis were viable.

4. Summary and Perspectives

The use of botanical products is very popular in the United States. One in four Americans use a botanical as part of their primary healthcare with billions of dollars spent annually. Botanicals have been used to treat ailments from the common cold to depression. Considering the American herbal industry is just over a quarter century old and the fact there is little scientific evidence to support the safety, efficacy and mode of action of herbal products, its popularity is perplexing.

The United States Food and Drug Administration (FDA) does not require manufacturers of herbal products to provide either proof of safety or efficacy as long as they are not advertised or marketed as food or drugs that can diagnose, treat or prevent diseases. Coupled with issues concerning safety and efficacy, interactions with conventional medicine remain unknown.

In fact, the popular botanical, St John's wort, used for the treatment of depression interacts with a number of conventional therapies including prescription antidepressants decreasing their effectiveness (Henderson et al., 2002; Rahimi and Abdollahi, 2012). In a review focused on interactions between botanicals and conventional drugs, the authors stressed the importance of physicians asking their patients about their botanical use during drug prescription (Gardiner et al., 2008). Studies have shown that botanicals can modulate gene transcription in biological systems (Shay and Banz, 2005; Watanabe et al., 2001) and botanicals may induce

gene transcription by serving as ligands to nuclear transporters (Moore et al., 2000; Urizar et al., 2002). Importantly, each botanical studied exhibits its own signature interactions.

Curcumin is one of several botanicals that is believed to possess antioxidant properties, and thus, protect cells against the damaging effects of reactive oxygen species (ROS). Oxidative stress caused by an excess increase in ROS has been linked to various diseases such as cancer, cardiovascular disease and diabetes. As such, curcumin has been touted as a therapeutic agent for the treatment of many common diseases (Anand et al., 2008; Hatcher et al., 2008)

Dictyostelium discoideum has proven to be an excellent non-mammalian model for the study of human disease and pharmacogenetics (Alexander and Alexander, 2011; King et al., 2009). To evaluate the fundamental effects of botanicals on cells, we focused our attention on the effects of curcumin on growth, cell physiology and the underlying molecular mechanisms of action.

Inconclusive studies have reported concentration levels of curcumin found in plasma to be in the nanomolar range (Garcea et al., 2005; Sharma et al., 2004) whereas curcumin's therapeutic doses have been reported in the micromolar range (Aggarwal and Harikumar, 2009). In our study, we demonstrated that curcumin has a dose dependent inhibitory effect on *D. discoideum* cell proliferation and 27 µM was enough to have lasting inhibitory effects on the cell.

Antioxidant properties have been attributed to curcumin (Ak and Gulcin, 2008) (Menon and Sudheer, 2007) and so it was of interest to determine how the antioxidant genes, superoxide dismutase and catalase responded to curcumin. We showed that curcumin negatively regulates transcription of some of the 7 SOD genes (*sod*A, *sod*B and *sod*2) and catalase A, raising questions about the antioxidant properties attributed to the botanical. It is interesting to note at this point that at concentrations found in plasma, vitamin C and beta-carotene may act as antioxidants, however at higher concentrations they may act as pro-oxidants (Rietjens et al., 2002). Since bioavailabity of curcumin is low (Prasad et al., 2014), it will be interesting to treat *D. discoideum* cells with curcumin at concentrations in the nanomolar range to see if curcumin does exhibit antioxidant properties and that concentration itself is a mechanistic regulator.

To investigate the global transcriptional changes induced by curcumin on *D. discoideum* we performed RNA sequence analysis. We demonstrated that curcumin does modulate transcription of about 600 genes - 5% of the genome. Subsequently, a thorough study of the genes affected will need to be done to provide further insight into proteins that are affected and their physiological role in the cell. The availability of a fully sequenced *D. discoideum* genome and documented success of utilizing RNA sequencing in transcriptional profiling makes *D. discoideum* an attractive model to study the global response to polyphenols and botanicals. This would be especially beneficial in identifying common genes and biological processes regulated by botanicals. A related species, *Dictyostelium purpureum*, is highly resistant to

curcumin even at concentrations 6-fold higher than what we observed to inhibit growth of wild-type *D. discoideum*. A similar RNA-seq analyses could help shed more light on genetic mechanisms underlying curcumin resistance by comparing the transcription profiles of both species after treatment with the botanical.

The RNA-seq analyses showed the up-regulation of genes involved in oxidoreductase activity in both early and late responses to curcumin. The biological process of reduction-oxidation (redox) involves the transfer of electrons from one molecule, the oxidant to another, the reaction catalyzed by a class of enzymes known as oxidoreductases. It will be important to elucidate how curcumin regulates the expression of these oxidoreductase activity genes and whether there are common regulatory mechanisms.

What we have been able to demonstrate from our studies using this relatively simple microbial system are the possible deleterious effects posed by botanicals. Despite over 6000 curcumin citations in the Library of Medicine, there is no conclusive scientific evidence demonstrating a primary target for curcumin. Instead, we observe modulation of hundreds of genes, all involved in complex biological processes in the cell.

The results from our studies are not meant to refute nor undermine the potential health benefits of curcumin, however, they do underscore the importance for further scientific research to establish curcumin's risks and benefits. Presently, the sale and

use of curcumin is based not only on inconclusive evidence as it relates to its safety and efficacy but also the lack of properly acknowledging potential harmful effects of the popular botanical. As a result of both the lack of scientific evidence and regulation surrounding botanicals, users of curcumin are poorly equipped to make decisions about using these as part of their primary healthcare.

Discovery of molecular mechanisms underlying drug resistance has led to better drug efficacy and ultimately led to improving the quality of life in the face of disease. Acriflavine is a topical antiseptic used to treat infected wounds and burns. It has recently been shown to inhibit HIF-1 preventing angiogenesis (Lee et al., 2009). As such, acriflavine is thought to have anti-cancer potential. Acriflavine was previously used in *D. discoideum* as a marker for parasexual genetic studies (Williams et al., 1974). Acriflavine resistance was mapped to four loci with catalase A being the only identified protein product encoded by any of the 4 loci (*acr*A).

In an attempt to identify and characterize genes conferring resistance to acriflavine, we took advantage of the widely-used biomedical model, *D. discoideum* and the availability of well-established molecular and genetic tools. Using an insertional mutagenesis approach, we generated 5 putative acriflavine resistance strains and identified the genes involved in acriflavine resistance in four of the strains.

Our ability to isolate mutants by insertion of exogenous DNA got us close to assigning biological significance to uncharacterized genes. Successful disruption of the

identified genes by homologous recombination would have allowed us to unequivocally link the identified genes to acriflavine resistance.

Understanding the complex mechanisms associated with drug response is difficult using mammalian models. The maintenance and care of such models as well as the large numbers of animals required for experimental study have necessitated the use of simple and genetically tractable non-mammalian models for initial studies.

Our studies prove the power of *D. discoideum* in understanding biological processes that can be translated to meaningful human applications. Despite the evolutionary distance between *D. discoideum* and humans, studies including those described in this work are assigning biological roles to genes conserved between social amoeba and humans, and suggest further avenues for their study.

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