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MITOCHONDRIAL FUNCTIONS ARE MAJOR TARGETS OF ISOCYANIDE ACTIVITY IN *SACCHAROMYCES CEREVISIAE*

by

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MITOCHONDRIAL FUNCTIONS ARE MAJOR TARGETS OF ISOCYANIDE ACTIVITY IN *SACCHAROMYCES CEREVISIAE*

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University of Nebraska, 2022

Advisor: Wayne R. Riekhof

The isocyanide functional group is important in the synthesis of many organic compounds and is found in natural products produced by plants, bacteria, marine invertebrates, and fungi. The antimicrobial activities of isocyanide compounds have been documented for almost 70 years, however, the biochemical targets and mechanisms of action remain poorly defined. We report antimicrobial activity of 4-*para*-nitrophenyl-isocyanide (*p*-NPIC) against a model fungus, *Saccharomyces cerevisiae*, and the human fungal pathogen *Candida albicans*. To identify the cellular and molecular targets of *p*-NPIC, we screened the non-essential single gene-deletion collection of *S. cerevisiae*. We aimed to identify genes which, when absent, rendered the resulting strain incapable of growth on solid media containing 1.5 μM *p*-NPIC. We identified 167 strains that were hypersensitive to *p*-NPIC and determined the minimum inhibitory concentration of *p*-NPIC for each of these mutant strains. The most sensitive deletion-strains (MIC < 3.0 μM in liquid media) were enriched in mitochondrial functions including the mitochondrial type II fatty acid synthase, lipoic acid biosynthesis and protein lipoylation, synthesis and assembly of iron-sulfur clusters, and assembly and maintenance of cytochrome c oxidase. The identification of essentially all components of the proton-pumping vacuolar membrane ATPase, as well as some pH sensitive components of membrane lipid

biosynthesis, also suggest a role for regulation of cytoplasmic pH as a key determinant of *p*-NPIC tolerance. Taken together, these results suggest that mitochondrial metal homeostasis and reactive oxygen scavenging are disrupted by *p*-NPIC treatment and provide new information about the potential mechanisms of action of isocyanide natural products.

Acknowledgements

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TABLE OF CONTENTS

List of Figures

List of Tables

CHAPTER 1: Structure, Function, Biosynthesis, and Degradation of Isocyanide Natural Products

Introduction

Isocyanides (also called isonitriles or carbylamines) (Santra et al., 2016) are organic compounds with the functional group made up of a carbon atom triple bonded to a nitrogen atom (**Figure 1.1**). Isocyanides are produced as secondary metabolites in several different groups of organisms and are involved in a wide variety of functions. There are currently over 200 isocyanide natural products that have been discovered. (Huang et al., 2020). The first naturally occurring isocyanide compound, xanthocillin, was isolated from *Penicillium notatum* in 1950 (Lim et al., 2018). There are both terrestrial (bacteria, fungi, and plant) (Massarotti et al., 2021) and marine organisms (sponges and mollusks) that produce isocyanides (Anjum et al., 2016; Manzo et al., 2004), with marine organisms responsible for synthesis of 63% of the naturally occurring isocyanide products that have been described (Emsermann et al., 2016). Isocyanides were first chemically synthesized in 1859, by forming allyl isocyanide from allyl iodide and silver cyanide (Massarotti et al., 2021). Additionally, one of the most notable aspects of the isocyanide group is that several of the low molecular weight compounds in this group have a particularly volatile, pungent smell (Duque et al., 2001).

Due to their electron rich molecular structure and presence of a stable carbanion (Harris et al., 2018), these natural products can serve a variety of purposes, including playing a crucial role in transition metal acquisition and acting as virulence factors in pathogenic organisms (Harris et al., 2018). Along with these characteristics, due to their structure and binding properties, isocyanides have potential as a new class of antimicrobials with activity against both bacteria and fungi. This literature review will

focus on the different classes of isocyanides, the biosynthesis (via isocyanide synthases, non-heme iron-dependent oxidases, and nonribosomal peptide synthetases) and degradation of isocyanides, and finally what is currently understood about the mechanism of action of isocyanide-containing compounds.

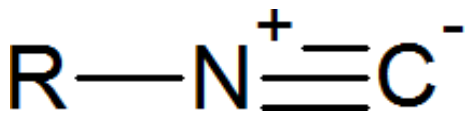

Figure 1.1: The isocyanide (also referred to as isonitrile/carbylamine) functional group.

Types of Isocyanides

Naturally occurring isocyanide products can be categorized into two main groups based on the organisms that make them: terrestrial isocyanides and marine isocyanides. Terrestrial isocyanide natural products are most commonly produced by bacterial and fungal species found in the soil, as well as from a few plant sources (Pedras & Park, 2016). Terrestrial isocyanides traditionally have the backbone of an aromatic alpha amino acid such as phenylalanine, tyrosine, or tryptophan. (Lim et al., 2018). In the terrestrial isocyanide group, the two main types of natural products are: cyclopentyl isocyanides and xanthocillin-type isocyanides (**Figures 1.2A & 1.2B**) (Edenborough, 1988). Marine isocyanides are produced by marine sponges and mollusks. Unlike terrestrial isocyanide products, marine isocyanide products are usually produced from terpenoids (Schnermann & Shenvi, 2015). In the marine isocyanide group, there are several different subclasses, but two of the largest are the diterpenes as well as sesquiterpenes (**Figure 1.3A & 1.3B**) (Garson & Simpson, 2004). This literature review will be focused mainly on terrestrial isocyanide products.

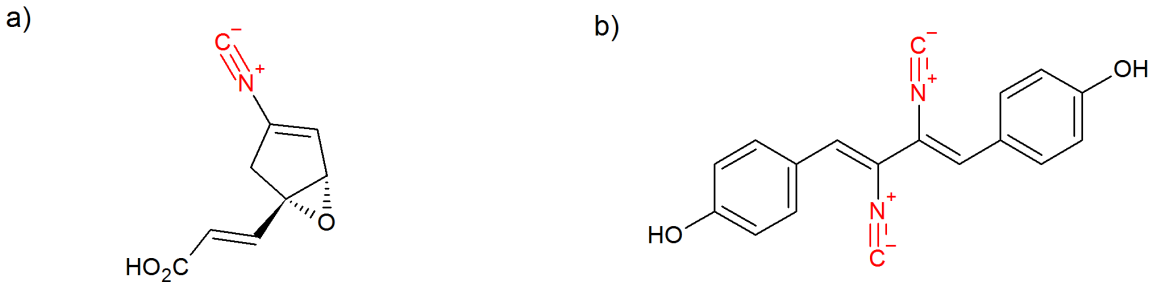

Figure 1.2: Examples of two types of naturally occurring terrestrial isocyanide products. a) A type of cyclopentyl isocyanide, dermadin, produced by fungal species of the genus *Trichoderma*. b) Xanthocillin, produced by *Aspergillus fumigatus* and *Penicillium notatum* the compound from which the group xanthocillin isocyanides got their name.

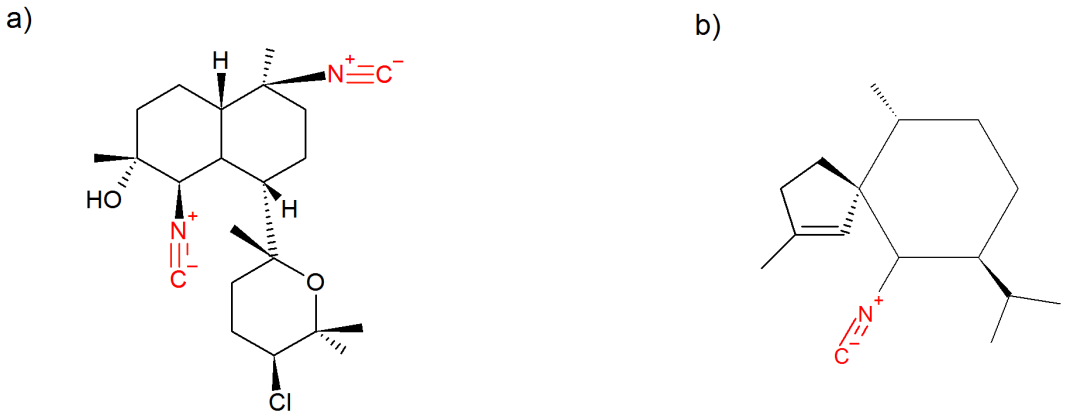

Figure 1.3: Examples of two types of naturally occurring marine isocyanide products. a) A type of diterpene isocyanide, kalihinol E produced by several *Acanthella* species. b) A type of sesquiterpene isocyanide, spiroxane produced by the species of the sea sponge genus *Hymeniacidon*.

Terrestrial Isocyanide Natural Product Biosynthesis

Synthesis of Terrestrial Isocyanides via Isocyanide Synthases

Currently, there are three known mechanisms by which terrestrial isocyanides are synthesized, the first of which uses isonitrile synthases (Huang et al., 2020). There are many different types of isonitrile synthases found in different organisms, which have been discovered over the past 17 years. One such isonitrile synthase is *isn*A, which was heterologously expressed from environmental DNA (eDNA) in *E. coli* (Brady & Clardy, 2005). The isonitrile synthase *isn*A works by converting an aromatic alpha amino acid group, using ribulose-5-phosphate as a co-substrate, to generate a product containing the isocyanide functional group (Chang et al., 2017). More specifically, *isn*A removes the carbonyl carbon from the ribulose-5-phosphate to create the triple bond between the nitrogen and carbon atoms in the isocyanide functional group (**Figure 1.4**).

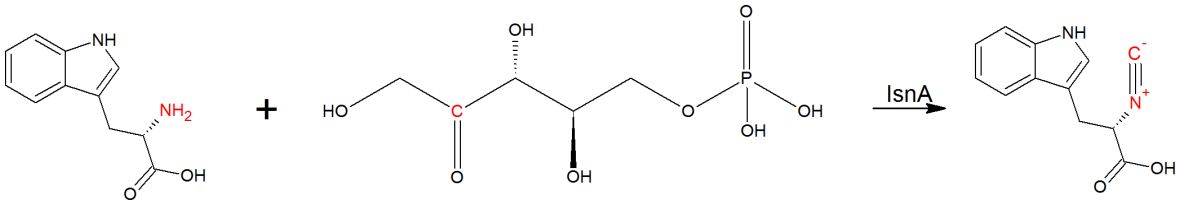

Figure 1.4: Formation of an isocyanide compound from an aromatic alpha amino acid (tryptophan) and ribulose-5-phosphate via *isn*A (Chang et al. 2017). *isn*A takes the carbonyl carbon from ribulose-5-phosphate and incorporates it using the amino group in tryptophan to make the isocyanide functional group.

*isn*A is not the only isocyanide synthase that has been discovered in microbes. Brady et al. sequenced several other bacterial genomes from eDNA and found several DNA sequences homologous to *isn*A (Brady et al., 2007).

One of these homologs were the genes *pvc*A and *pvc*B. The *pvc* gene cluster (A, B, C, and D) was first found in the bacteria, *Pseudomonas aeruginosa*, and is required for biosynthesis of the natural product pyoverdine (Clarke-Pearson & Brady, 2008). Pyoverdine acts as a virulence factor in *P. aeruginosa*, via its metal chelating abilities (Braud et al., 2009), which are common with isocyanide natural products. Two other *isn*A homologous gene clusters are: *amb* (*ambI*1 and *ambI*2), and the *wel* gene cluster found in the cyanobacteria species *Westiella intricata* (Micallef et al., 2014). Both the *amb* and *wel* gene clusters are responsible for a wide variety of marine isocyanide products, which have a variety of functions including broad spectrum antimicrobial activities as well as insecticidal activities, also corresponding with their ability to chelate metals (Becher et al., 2007; Mo et al., 2009).

Fungi have also been found to synthesize isocyanide natural products via isocyanide synthases. *Aspergillus fumigatus* encodes a biosynthetic gene cluster (BGC) including an isonitrile synthase involved in xanthocillin production (Lim et al., 2018). These genes are named *xan* (A-G) and are regulated by the copper-binding transcription factors *ace*A and *mac*A (Raffa et al., 2020) that regulate copper homeostasis. When extracellular copper levels are decreased, *ace*A (a component of the copper detoxification system) and *mac*A (aids in copper uptake during copper starvation) transcription levels are increased. Along with the *xan* genes, Lim et al. also discovered another isocyanide synthase in other species of fungi by comparing the amino acid

sequences of the *pvc* gene cluster (Clarke-Pearson & Brady, 2008) and the *isn* gene cluster (Brady & Clardy, 2005) to the genome of *A. fumigatus* (2018). The resulting isocyanide synthase found was a copper-responsive metabolite *crm*A. Which, similarly, to *xan* genes is regulated by *mac*A but not *ace*A.

Synthesis of Terrestrial Isocyanides via Nonheme Iron (II)-Dependent Oxidases

Isocyanide synthases have been the most common way organisms synthesize isocyanide natural products, but more recently other biosynthetic mechanisms have been discovered. The second pathway of isocyanide formation is via a nonheme iron (II)-dependent oxidases/decarboxylases (Harris et al., 2018). This pathway was discovered through genome mining of conserved clusters of genes found in many species of the phylum Actinobacteria, but particularly the species *Streptomyces coeruleorubidus*. Harris et al. found a cluster of 5 genes, *sco*A-E, which synthesizes isonitrile lipopeptides (INLPs) in *S. coeruleorubidus* (2018). INLPs play an important role in metal homeostasis in many species of Actinobacteria (Harris et al., 2017), which reinforces the hypothesis that isocyanide compounds play an important role in the acquisition and regulation of metals both inside and outside the cell. Of this cluster of genes, *sco*E plays the important role of forming the intermediate compound which contains an isocyanide functional group. The enzyme *sco*E has been identified as a nonheme iron (II)-dependent oxidase, which plays a very similar role to isonitrile synthases in the synthesis of natural isocyanide products. *sco*E takes the carbonyl carbon from a free acid substrate and transfers it to an aromatic alpha amino acid to form the isocyanide functional group (Harris et al., 2018).

Synthesis of Terrestrial Isocyanides via Nonribosomal Peptide Synthetases

The third biosynthetic pathway of isocyanide natural products uses a nonribosomal peptide synthetase. Much like the other methods used to synthesize these compounds, this mechanism was discovered through metagenome sequencing and bioinformatic analysis. One example of nonribosomal peptide synthetase in the production of isocyanide products is from Wang et al., where the genome of *Streptomyces thioluteus* was sequenced and 40 poorly studied biosynthetic gene clusters were found (2017). Many of those gene clusters included another isocyanide-producing gene cluster based on nonribosomal peptide synthetases (NRPS) named *sfa*. The *sfa* gene cluster (A-F) was found to synthesize a diisonitrile product called SF2768 in *S. thioluteus* (Amano et al., 2011) which, like many of the previous isocyanide natural products referenced here, is thought to be a regulator of metal homeostasis, specifically acting as a copper chelator (Wang et al., 2017). *sfa*A is a putative dioxygenase, *sfa*BCD are the NRPS-related enzymes, *sfa*E is a hydroxylase, and *sfa*F (previously called Orf23) is an isocyanide hydratase, which will be discussed in a later section. Although, the exact mechanism of action of *sfa*A is still unknown, it is thought to be involved with the biosynthesis of the 3-isocyanobutanoic acid building blocks, which contain the isocyanide functional group (Wang et al., 2017). This building block is then used in further intermediate reactions by *sfa*BCD to finally synthesize SF2768.

Isocyanide Degradation via Isocyanide Hydratases

Preliminary Work on Isocyanide Degradation

The enzyme isocyanide (isonitrile) hydratase catalyzes the cleavage of the triple bond between the nitrogen and carbon atoms in the isocyanide functional group. Before the discovery of the isocyanide hydratase, the only other known enzyme that interacted with isocyanide compounds, specifically methyl isocyanide, was nitrogenase (Goda et al., 2001). Nitrogenase, using a reduction reaction, could break down the triple nitrogen-carbon bond into either dimethylamine (Kelly et al., 1967), or methylamine and methane (Rubinson et al., 1983).

Before the isocyanide hydratase enzyme was first identified, it was thought that N- substituted formamides were the precursor to isocyanide compounds. This was because in organisms that synthesize isocyanide compounds, there was a corresponding N-substituted formamide found with it (Burreson & Scheuer, 1974). The N-substituted formamide to isocyanide pathway was challenged by Hagadone et al. where it was shown that in the sea sponge genus, *Hymeniacidon*, the reaction that occurs produces an N-substituted formamide and isothiocyanate as the products from the isocyanide compound isocyanopupukeanane (1984). However, the reaction was not found to occur in the opposite direction. Along with this observation, the discovery of the first isocyanide hydratase (Goda et al., 2001) also strengthened the isocyanide compound to the N-substituted formamide hypothesis for degradation of isocyanide products.

Discovery of Isocyanide Hydratases

The first isocyanide hydratase was identified in 2001, from the soil bacteria *Pseudomonas putida* strain N19-2 (Goda et al., 2001). To recover the isocyanide hydratase enzyme, Goda and colleagues cultured *P. putida* N19-2 for two months in the

presence of a compound, cyclohexyl isocyanide, which acted as the sole nitrogen source for the culture media. Several other isocyanide compounds were also used as a nitrogen source, to compare the differences between them. These compounds included: benzyl isocyanide, methyl isocyanoacetate, ethyl isocyanoacetate, and isocyanomethyl phosphonic acid diethyl ether (Goda et al., 2001). While the addition of some isocyanide compounds induced an increase in isocyanide hydratase activity, cyclohexyl isocyanide treatment induced the highest levels of enzymatic activity.

Furthermore, unlike other isocyanide compounds, when cyclohexyl isocyanide was added to the culture, the authors noted that another product was formed via the hydration of the isocyanide group by water (**Figure 1.5**). Given this mechanism the authors named this new enzyme isocyanide *hydratase*. The resulting product was identified via mass spectrometry to be an N-substituted formamide, *N*-cyclohexylformamide. This enzyme was then specifically named cyclohexyl-isocyanide hydratase (InhA) (Lakshminarasimhan et al., 2010) to reflect the specificity of this enzyme. Without the addition of an isocyanide compound to the culture, there was no isocyanide hydratase activity detected. This strongly suggests that isocyanide hydratase activity is regulated dynamically with regard to nitrogen availability and the presence of isocyanide compounds. Isocyanide hydratases are the cell's defense mechanism in response to extracellular isocyanide compound presence. The synthesis of isocyanide hydratase is likely a costly energy process, and therefore is only needed by the cell when there is an isocyanide compound in the surrounding environment.

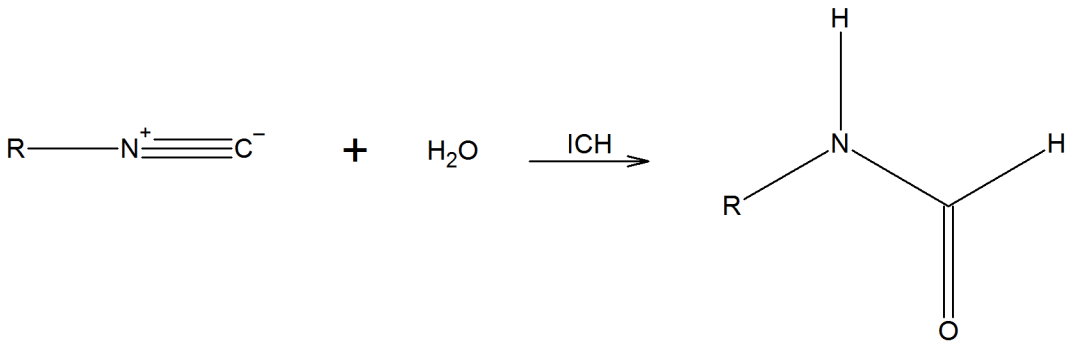

Figure 1.5: Model hydration reaction between an isocyanide compound and water via isocyanide hydratase (ICH), resulting in an N-substituted formamide.

Other Isocyanide Hydratases

A few years after the discovery of the first isocyanide hydratase, a second isocyanide hydratase was discovered in another soil organism, *Arthrobacter pascens* strain F164 (Fukatsu et al., 2004). The method of identifying this isocyanide hydratase was very similar to the process above, but instead of using cyclohexyl isocyanide in the culture media, benzyl isocyanide, and later, N-benzylformamide were used instead. Fukatsu et al. discovered in *A. pascens* that there is a second enzyme that catalyzes hydrolysis of the N-formamide created by isocyanide hydratase (2004). This enzyme was named N-substituted formamide deformylase (NfdA) and was able to degrade the N- substituted formamide into amines and formate (Fukatsu et al., 2004). The amines and formate can then be used for other cellular processes, which demonstrates that not only is *A. pascens* able to synthesize an isocyanide hydratase that can break down toxic isocyanide compounds, but it can then use the products of that reaction (using NfdA) to benefit itself.

Another study by Sato et al. was conducted to compare the isocyanide hydratases from *P. putida* and *A. pascens* (2010). They showed that while both isocyanide hydratases were able to degrade isocyanide products, the fact that the *A. pascens* had a second enzyme, NfdA, present suggested that the two hydratases may have different mechanisms of action. Furthermore, both primary and secondary structures of both hydratases differ significantly from one another. The fact that there was no discernable sequence identity between the two hydratases, suggest that the isocyanide hydratase found in *A. pascens* was a novel isocyanide hydratase, and subsequently named it InhB (Sato et al., 2010).

Isocyanide hydratases have also been discovered in other organisms including *Pseudomonas fluorescens* strain pf-5 and *Streptomyces thioluteus* (Lakshminarasimhan et al., 2010; Zhu et al., 2018). *P. fluorescens* was found to have a 36% protein sequence similarity to the original InhA, found in *P. putida*. The *S. thioluteus* isocyanide hydratase is sfaF (previously called Orf23 from the *sfa* gene cluster) (Zhu et al., 2018) previously mentioned and is part of the BGC for synthesis of terrestrial isocyanides via nonribosomal peptide synthetase. As Zhu et al. suggest, more isocyanide hydratases could be found in the future via genome mining (2018). Given the ubiquity of hydrolytic enzymes of different mechanisms, we might expect to find more examples of convergent evolution of isocyanide hydratase function, as noted above for the enzymes from *P. putida* and *A. pascens*.

Mechanism of Action of Isocyanide Compounds – Transition Metal Chelators

The isocyanide structure has been described as electron rich (Harris et al., 2018), and can serve as either a nucleophile or an electrophile in nucleophilic substitution reactions (del Mar Conejo et al., 1999). Isocyanide compounds thus have many interesting chemical properties that make them a building block of many different organic synthesis processes. Coupled with the fact that a diverse range of organisms are capable of synthesizing isocyanide compounds (Anjum et al., 2016; Manzo et al., 2004; Massarotti et al., 2021), there is a wide range of functions that isocyanide compounds can perform.

One of the most notable characteristics is that isocyanide products frequently act as transition metal chelators (Amano et al., 2011; Braud et al., 2009; Harris et al., 2017; Lim et al., 2018). Specifically, isocyanide compounds are often either a copper or iron chelator, both of which are important to prokaryotic and eukaryotic cellular functions (Cairo et al., 2006; Ruiz et al., 2021). The strength of the iron-isocyanide interaction can be explained by an analogy to carbon monoxide. The isocyanide functional group is isoelectric with carbon monoxide, which is a high affinity heme ligand (Massarotti et al., 2021). Generally, the transition metals bond with the carbon atom from the isocyanide functional group, but they can also bind with the nitrogen atom, or both the carbon and nitrogen atoms (**Figure 1.6**) (del Mar Conejo et al., 1999; Massarotti et al., 2021).

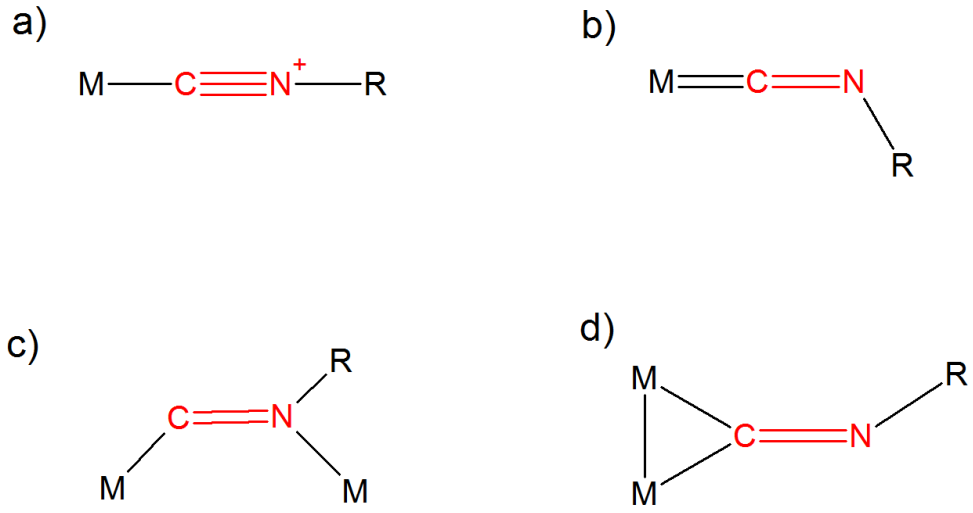

Figure 1.6: Metal-isocyanide bonding interactions, adapted from Massarotti et al., 2021. Due to the structure of the isocyanide functional group, transition metals tend to bond to the carbon atom (a, b, and d), but also can bond to the nitrogen atom (c) as well.

The interactions between the isocyanide functional group and these transition metals results in a variety of functions, such as antimicrobial properties. One example of this phenomenon is the diisocyanide product SF2768, from the organism *Streptomyces thioluteus* (Wang et al., 2017). SF2768, like many other isocyanide products is a metal chelator, more specifically a copper chelator. Once synthesized, *S. thioluteus* transports SF2768 outside of the cell, where it binds to extracellular copper to form a complex. The complex is then transported back inside the cell where the copper can be used for different cell processes (Wang et al., 2017). SF2768 acts as an antimicrobial agent by removing extracellular copper from the environment, therefore removing the ability of surrounding organisms to utilize that copper, while also being able to uptake the copper for its own benefit. A later paper by Zhu et. al found that in the presence of SF2768, several species of bacteria including *Acinetobacter baumannii* and *Bacillus subtilis* experienced copper deficiency and showed an increase in reactive oxygen species (ROS), which induced growth inhibition (2020). This further supports the hypothesis that SF2768 acts as a copper chelator. A similar copper chelating effect can be seen in xanthocillin (Raffa et al., 2021), which is produced by both *Penicillium notatum* and *Aspergillus fumigatus* (Lim et al., 2018). Not only does xanthocillin have binding properties to copper, but it has also been reported to have antimicrobial properties through heme binding as well (Hübner et al., 2021).

Along with antimicrobial properties, isocyanide natural compounds also can act as virulence factors in pathogenic microbes. One example of this functionality is the isocyanide product rhabduscin, which is produced by insect and nematode pathogens: *Photorhabdus luminescens* and *Xenorhabdus nematophila* (Crawford et al., 2012).

Rhabduscin is yet another copper chelating isocyanide compound which inhibits a copper-dependent enzyme in both insects and nematodes called phenoloxidase. Both use phenoloxidase to synthesize melanin, which acts as an antimicrobial defense for the organism (Correa et al., 2017). By disrupting the synthesis of melanin, rhabduscin allows the pathogens to take over the insect or nematode more effectively.

Conclusions and Future Work

There are a wide variety of organisms which synthesize isocyanide compounds, ranging from microbes, sponges, mollusks, and plants. Each of these different compounds can serve a wide variety of purposes including transition metal acquisition, antimicrobial activities, and even acting as virulence factors in pathogenic organisms. Each of these mechanisms, as well as the biosynthesis of isocyanide products, is highly dependent on extracellular metal concentration. Given the variety of isocyanide compounds and their specific functionalities, isocyanide products need to be evaluated individually for their unique functions.

A synthetic isocyanide compound, para-nitrophenyl isocyanide (*p*-NPIC), was developed by the UNL labs of Drs. Mark Wilson and David Berkowitz as a "model" isocyanide compound to better understand the mechanisms of isocyanide hydratases (Dasgupta 2020). We decided to determine whether this model isocyanide had antimicrobial properties similar to isocyanide natural products. We also wanted to determine the mechanism of action of any antimicrobial activities of this model isocyanide compound, and how it compares to naturally occurring compounds and this work is the subject of Chapter 2 of this thesis. With the wide variety of functions ascribed

to the isocyanide functional group, there could be several human health related activities, particularly as an antifungal compound, given the rise in prevalence of pathogenic fungi that are resistant to first-line antifungal drugs.

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CHAPTER 2: Mitochondrial Functions are Major Targets of Isocyanide Activity in *Saccharomyces cerevisiae*

Introduction

The isocyanide organic functional group, also referred to as isonitrile, has long been used as a building block in a number of organic synthesis processes, owing to its unusual electronic properties (Yurino & Ohkuma, 2020). Isocyanide functional groups also occur in diverse natural products of bacterial (Brady & Clardy, 2005; Massarotti et al., 2021; Wang et al., 2017), fungal (Lim et al., 2018; Raffa et al., 2020), marine sponge (Anjum et al., 2016), and plant (Pedras & Park, 2016) origin. The first discovered isocyanide natural product, xanthocillin, features two isocyanide moieties that are critical for its potent antimicrobial activity (Lim et al., 2018). Studies over the last 15 years have identified multiple genes encoding microbial isocyanide synthases which make isocyanides (Brady et al., 2007; Brady & Clardy, 2005), isocyanide hydratases which degrade them (Fukatsu et al., 2004; Goda et al., 2001; Lakshminarasimhan et al., 2010; Sato et al., 2010), and the biochemical pathways for the synthesis of a number of isocyanide compounds (Amano et al., 2011; Harris et al., 2017, 2018; Wang et al., 2017). The biological functions of isocyanide natural products are mostly unknown, however, several natural products act as antibiotics that presumably benefit the producing organism (Harris et al., 2017; Hübner et al., 2021; Zhu et al., 2018).

Because isocyanides can be potent antibiotics with low toxicity to mammalian cells (Massarotti et al., 2021), there is considerable interest in identifying their mechanism of action for future use in pharmaceuticals. A recent study has shown that xanthocillin dysregulates heme biosynthesis by directly binding to heme and sequestering it from its target hemoproteins, providing a rare example of an antibiotic that acts on an essential cofactor rather than a target biomacromolecule (Hübner et al., 2021). The

avidity of the isocyanide-heme interaction has been long-appreciated by hemoprotein biochemists, where isocyanides have been extensively used *in vitro* to inhibit heme-dependent reactions. The strength of the iron-isocyanide interaction is due, in part, to isocyanides being isoelectronic with carbon monoxide, another notoriously high-affinity heme ligand (Massarotti et al., 2021). Broadening the connection between isocyanides and transition metals, the isocyanide natural product rhabduscin is a potent inhibitor of the copper-dependent enzyme phenoloxidase. Rhabduscin is produced by certain gram-negative bacteria that colonize nematodes, where inhibition of phenoloxidase decreases the ability of the nematode to synthesize melanin as a defense against microbial colonization (Crawford et al., 2012). In addition, recent studies indicate that some diisocyanides (Wang et al., 2017) function as copper-chelating siderophores or "chalkophores" that are important for mediating copper acquisition and moderating its toxicity. The diversity of potential targets for isocyanide natural products suggests that isocyanides can be used for a variety of purposes.

Due to the unique properties of isocyanides and their highly specific properties, each individual isocyanide product needs to be evaluated for its unique functionalities. In this chapter, we aimed to see if the chemically synthesized isocyanide product, para-nitrophenyl-isocyanide, had antimicrobial properties, with particular focus on anti-fungal properties. We also wanted to determine the mechanism of action of any antimicrobial activities of this model isocyanide compound, and how its mechanism of action compares to naturally occurring isocyanide compounds.

Materials and Methods

Strains and Growth Conditions

Several strains were used for this study and can be found in **Table 2.1**. *Saccharomyces cerevisiae* and *Candida albicans* strains were grown in both yeast peptone dextrose (YPD) media (yeast extract (10 g/L), peptone (20 g/L), and dextrose (20 g/L)), and synthetic complete (SC) media (yeast nitrogen base without amino acids, carbohydrate, or ammonium sulfate) (1.7g/800 mL), ammonium sulfate (5g/800 mL), 2% w/v dextrose, and 1% v/v 10X complete amino acid mixture. *Escherichia coli* and *Bacillus thuringiensis* were grown in LB broth media (25 g/L) from Fisher BioReagents (#BP1426-2). For all media recipes, if the media needed to be solid, 20 g/L of agar was added. Liquid cultures were seeded as follows: 5 mL of preferred media in a 25 mL Erlenmeyer flask grown in a shaking incubator at 200 rpm at desired temperature.

Table 2.1 List of strains and corresponding genotypes of organisms used in this study.

Organism	Strain	Description
Saccharomyces cerevisiae	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
Saccharomyces cerevisiae	SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801*
Candida albicans	SN152	*arg4Δ/arg4Δ leu2Δ/ leu2Δ his1Δ/ his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434*
Escherichia coli	XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tetr)*]
Bacillus thuringiensis	HD-73	*Subspecies Kurstaki*

Synthesis of p-nitrophenyl isocyanide

Synthesis of *p*-nitrophenyl isocyanide was done by Dr. David Berkowitz's lab at the University of Nebraska – Lincoln, Department of Chemistry. The full synthesis protocol (including crystallization procedures) can be found in Dasgupta, 2020. *p*-nitrophenyl isocyanide synthesis was completed in two separate steps. The first step was the synthesis of N-(4-Nitrophenyl) formamide in which 10 mmol of amine and 12 mmol of formic acid was brought to 60 °C and stirred for 2 hours. After verification N-(4-Nitrophenyl) formamide using ^{1}H NMR, ^{13}C NMR, IR, and Time of Flight (TOF) mass spectra, we could proceed to the second step of the synthesis process.

To complete the synthesis of *p*-nitrophenyl isocyanide, 8 mmol of N-(4-Nitrophenyl) and 56 mmol of triethylamine were dissolved in 30 mL of CH_2Cl_2 and then cooled to -10 °C. The same verification procedures for N-(4-Nitrophenyl) were performed to confirm synthesis of *p*-nitrophenyl isocyanide. *p*-nitrophenyl isocyanide was kept at -80 °C as a solid until it was needed for experimentation. When used for experimentation, *p*-nitrophenyl isocyanide was diluted to 10 mM in dimethyl sulfoxide (DMSO) and kept at -80 °C, with more dilute working stocks (1 mM) being kept at -20 °C.

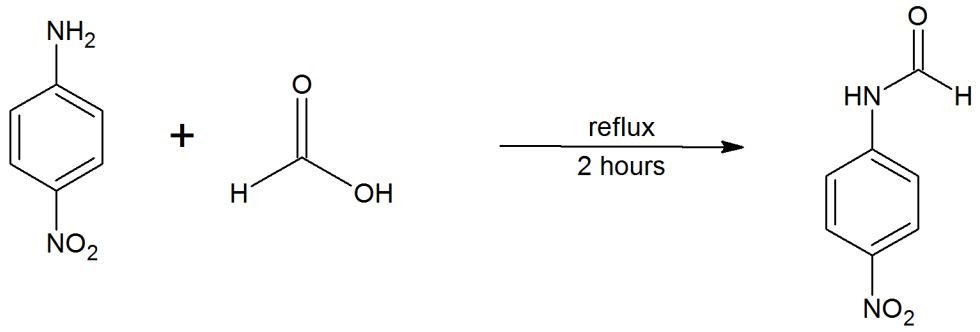

Figure 2.1 Synthesis of N-(4-Nitrophenyl) from Dasgupta (2020).

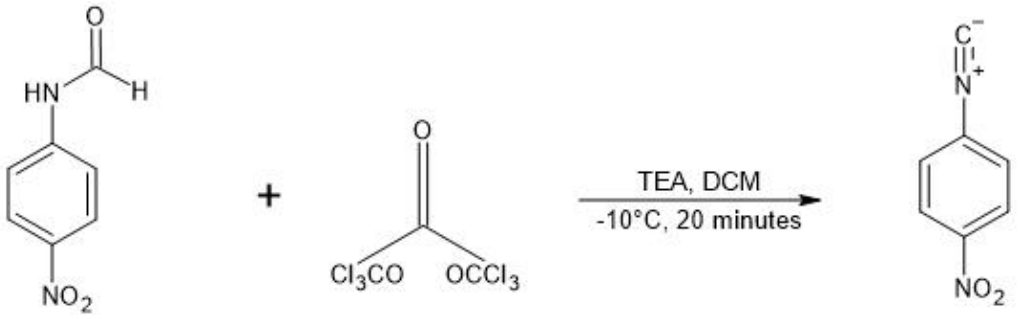

Figure 2.2 Synthesis of *p*-nitrophenyl isocyanide via the precursor N-(4-Nitrophenyl), from Dasgupta (2020).

Sensitivity testing of representative bacteria and fungi

S. cerevisiae (a generally non-pathogenic fungi), *C. albicans* (common pathogenic fungi), *E. coli* (gram-negative bacteria), and *B. thuringiensis* (gram-positive bacteria) were used to test the sensitivity of representative bacteria and fungi to *p*-nitrophenyl isocyanide. 100 µl of liquid overnight cultures (grown as stated above) of *S. cerevisiae*, *E. coli*, and *B. thuringiensis*, respectively, were diluted 1:100 in fresh media (either SC or LB) and were added to 100 µl of a 2-fold serial dilution of *p*-nitrophenyl isocyanide (ranging from 0 µM- 50 µM final concentration), in a flat bottom tissue cultured treated 96-well plate (Fisher Brand, #FB012931). *C. albicans* dilutions were done in the same manner but were instead grown in a flat bottom non-treated 96-well plate (Eppendorf, #0030730011). Each of the representative microbes were tested in triplicates. *S. cerevisiae*, *B. thuringiensis*, and *C. albicans* plates were grown at 30 °C for 24 hours, and the *E. coli* plate was grown at 37 °C for 24 hours. After incubation, the optical density (OD) of the plates was read at 600 nm using the Synergy H1 Hybrid Reader (BioTek, # 11-120-535).

Fungistatic vs Fungicidal

To determine if *p*-nitrophenyl isocyanide is a fungistatic or fungicidal compound survivorship curves were created. A 50 mL overnight culture of *S. cerevisiae* BY4741 in SC media was used to seed several 50 mL flasks at a starting OD_{600} of 0.1 in 100 µL of a flat-bottom 96-well plate. Flasks grown at 30 °C as described above and were allowed to grow to an OD_{600} of 0.2 (100 µL in a flat-bottom 96-well plate) to allow cells to reach exponential growth phase. Once flasks reached an OD_{600} of 0.2, three different

concentrations of *p*-nitrophenyl isocyanide (0 µM control, 3 µM, 4.5 µM, and 6 µM) were added to their respective flasks. One hour after the addition of *p*-nitrophenyl isocyanide, a 1 mL aliquot was taken from each flask and transferred to a 1.5mL microcentrifuge tube. The flasks were put back into the incubator so another aliquot could be taken from them at 24 hours. The aliquots were spun down for 1 minute at 10,000 xG in an accuSpin Micro 17 centrifuge (Fisher Scientific, #13-100-675) and the supernatant was removed. Samples were washed three times in 1X PBS. Samples were then serially diluted 1:10 in 1.5 mL microcentrifuge tubes containing 1X PBS from 10^{-1} – 10^{-6}. 100 µl from the 10^{-4} – 10^{-6} dilutions were spread onto YPD plates in triplicate (final dilutions 10^{-5} – 10^{-7}) and incubated overnight at 30 °C. The washing and plating process was repeated for the samples at the 24-hour mark. Colonies were counted by hand for all four *p*-nitrophenyl isocyanide concentrations and were recorded for both time points.

Genetic screen of gene-deletion collection for p-NPIC sensitive strains

The MATα non-essential gene deletion collection, from Brachmann et al. (1998) was used for the genetic screen for sensitivity to *p*-NPIC. The MATα plates were thawed, mixed with a stainless steel 96-pin blotter, and then diluted in a 96-well plate with 150 µl of fresh YPD media (~50-fold final dilution). The diluted cells were then pinned on either YPD media or YPD media + 1.5 µM of *p*-NPIC. Plates were grown at 30 °C for 48 hours, with growth being recorded every 24 hours. Colonies were deemed sensitive to *p*-NPIC if there was minimal, or no growth recorded after 48 hours. To confirm sensitivity to *p*-NPIC, mutants which were sensitive on solid YPD + 1.5 µM *p*-NPIC were then taken and grown on *p*-NPIC-free solid YPD media. Single colonies from these plates were taken and grown in liquid YPD overnight at 30 °C. A two-fold serial dilution of *p*-

NPIC (starting at 50 μM) was done in the same manner as described above. Growth was recorded after 24 hours, and mutants that were sensitive to *p*-NPIC in liquid culture were confirmed hits for the rest of the study.

Gene ontology analysis

Primary and secondary screening, on both solid and liquid media respectively, led to 167 sensitive mutants being found. Gene ontology (GO) analysis was performed using the tool YeastMine, which can be located on the Saccharomyces Genome Database website (https://www.yeastgenome.org/). Receiver-operating characteristic (ROC) analysis was performed on the hits from the genetic screen using YeastNet v3 (Kim et al., 2014). ROC analysis was performed to determine how connected the hits from the genetic screen were to one another. By evaluating the area under the curve (AUC) value from the ROC analysis, we can determine whether the hits were significantly related to one another or not.

Creation of mutants in alternative background strain

To ensure the results of the genetic screen were from the specific deleted gene and not an artifact of the background strain, verification of mutants in an alternative background strain needed to be performed. To verify the results, a small selection of genes from the initial hit list were transformed into a different *S. cerevisiae* background strain, SEY6210. Gene deletion mutants selected were: *gcv3* (part of the mitochondrial glycine decarboxylase), *oar1* (mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase),

and *nfu1* (involved in Fe-S cluster transfer to mitochondrial clients) (Saccharomyces Genome Database).

To transform the mutants, first genomic DNA was isolated from the BY4741 background strain mutants using the CTAB fungal DNA extraction protocol, which was adapted from Cubero et al. (1999). Then, PCR was performed to amplify the knockout construct portion of the mutants before introduction into the SEY6210 background strain. Primers for each knockout construct were constructed using the Primer-BLAST program from NIH (Ye et al., 2012). Primers were designed using 500 base pairs up and downstream of the knock-out (K.O.) construct, which contains a selection marker for aminoglycoside resistance. Primer sequences used in this study can be found in **Table 2.2**.

Table 2.2: List of primers used for transformation of *S. cerevisiae* strain BY4741 to *S. cerevisiae* strain SEY6210.

Target	Primer Sequence
Δgcv3 F	5'- TCT GGG TCC ATT CCT TGA AAA CA -3'
Δgcv3 R	5'-AGC AGC AAA CGA AAG TGG AA -3'
Δoar1 F	5'- TTC CAA CAA CAA CAG CAA CAA -3'
Δoar1 R	5'- AGC GTC GTA AGC ATC AGA CA -3'
Δnfu1 F	5'- AAG TAC CAC ACT CTA GCC GAA -3'
Δnfu1 R	5'- GGA GAG TGT TGG GGA CGT AG -3'

Each PCR reaction was compromised of 38 µl UltraPure Distilled Water (Invitrogen, #10977-015), 5 µl 10X PCR Rxn Buffer (Invitrogen, #18067017), 2.5 µl 50 mM $MgCl_2$ (Invitrogen, #18067017), 1 µl diluted dNTPs (Invitrogen, #10297018), 1 µl of 20 µM forward primer (IDT), 1 µl of 20 µM reverse primer (IDT), 0.5 µl Taq Polymerase (Invitrogen, #EP0401), and 1 µl target DNA for a total volume of 50 µl. PCR amplification was done on an MJ Mini Personal Thermal Cycler (BioRad, #PTC-1148). The PCR thermal profile ran for each of the mutants was: 1 cycle at 95 °C for 5 minutes, 34 repeated cycles at 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1.5 minutes, 1 cycle at 72 °C for 5 minutes, END. The QIAprep® Spin Miniprep Kit (Qiagen, #27104) was used to purify the final PCR product. PCR products were then run on a 1X TAE gel at 80 volts for 1 hour using a PowerPac Basic power supply (BioRad, #1645050) to ensure the correct product was amplified before transformation.

Transformations were done following the protocol from (Gietz & Schiestl, 2007). Transformants were plated on YPD + Geneticin (G418) (100 mg/mL) in triplicate and were incubated at 30 °C for two days. To ensure the yeast had taken up the K.O. construct, three colonies were taken from initial transformation plates and re-struck on fresh YPD + Geneticin plates. For storage of the transformed isolates, one transformed colony from each plate was grown in liquid YPD media for 24 hours at 30 °C. The next day 700 µl from the overnight cultures was added to a freezer stock tube containing 300 µl of 50 % glycerol and flash frozen using liquid nitrogen.

DNA was isolated from the transformant stocks using the same CTAB fungal extraction protocol stated above. PCR and gel electrophoresis were performed using the

same parameters as mentioned above to verify that the K.O. construct had been successfully transformed into the new background strain, SEY6210.

Verification of mutants identified in primary screen

One colony of the desired mutant from both BY4741 and SEY6210 background strains were selected from previously grown YPD plates and were incubated as described above at 30 °C for 24 hours. A 100 µl, 2-fold serial dilution of *p*-nitrophenyl isocyanide (starting concentration of 50 µM) was done as previously described. Curves and minimum inhibitory concentrations from both background strains were compared to ensure the genetic screen results were accurate and not a result of an artifact from the original background strain.

Copper Rescue

Several naturally occurring isocyanide products act as copper chelators (Lim et al., 2018; Raffa et al., 2020), but not all isocyanide compounds are. To identify if our synthetic isocyanide compound, *p*-nitrophenyl isocyanide, can also act as a copper chelator, we tested the effects of the addition of copper to *S. cerevisiae* cells challenged with a relatively toxic level of *p*-nitrophenyl isocyanide (10 µM). In 96-well plate concentrations of $CuSO_4$ were diluted between 1-10 mM. 100 µl of diluted cells were added, as previously described. The OD_{600} was taken after 24 hours of growth at 30 °C.

Reactive Oxygen Species (ROS) Assay

Since many of the hits from the genetic screen of *p*-NPIC were mitochondrially related, we wanted to see if *p*-NPIC induced an increase in reactive oxygen species (ROS). To test this, an assay was performed using the DCFDA/H2DCFDA Cellular ROS Assay Kit (abcam, #ab113851) on both background strains BY4741 and SEY6210. DCFDA dye, when introduced to the cell, binds with any reactive oxygen species that are being produced by the cell. A culture of each *S. cerevisiae* strain was grown for 24 hours at 30 °C in SC media. Cells were then normalized to an OD_{600} of 0.5, washed, and pelleted in a 1.5 mL microcentrifuge tube with the supernatant removed. 500 µl of 50 µM of DCFDA dye, diluted in the 1X buffer that came in the kit, was added to each of the cell pellets. The pellets were then resuspended and incubated in a dark incubator at 30 °C for 30 minutes. The cells were then washed 3 times with SC media, and varying concentrations of *p*-NPIC were added to each of the 1.5 mL microcentrifuge tubes. Cells were again resuspended and allowed to incubate with the *p*-NPIC for 1 hour. After incubation, cells were washed 3 times with 1X PBS buffer. 100 µl from each of the 1.5 mL microcentrifuge tubes was transferred to a black 96-well plate with a clear bottom (ThermoFisher, #165305). The fluorophore was read at 485/535 and the optical density was taken at 600 nm.

Results and Discussion

p-nitrophenyl isocyanide is a potent antifungal compound

Isocyanide compounds have been identified as having antimicrobial activities for almost 70 years (Lim et al., 2018) however the mechanisms by which these compounds inhibit microbial growth is understudied and is only now coming to light for certain chemical processes. To exploit bacterial and fungal functional-genomic resources in interrogating this question, we tested 4 different types of microbes against *p*-NPIC to see its effectivity. Both fungi tested, *S. cerevisiae* and *C. albicans*, were highly sensitive to *p*-NPIC. The minimum inhibitory concentration (MIC) for *S. cerevisiae* strain BY4741 was ~10.5 μM, while the MIC of *C. albicans* was ~25 μM as seen in (**Figure 2.3)**. The *C. albicans* strain was grown at 30 °C instead of 37 °C, as not to trigger biofilm formation (Nadeem et al., 2013), which may have a further effect on its sensitivity to *p*-NPIC, and would be of great interest for future experimentation.

The gram-negative organism, *E. coli* was surprisingly tolerant to higher levels of *p*-NPIC than either of the two fungi, with an MIC of ~50 μM (**Figure 2.3**). This is an unusual result, since (as will be discussed in a later result) our genetic screen reveals that *p*-NPIC largely effects mitochondrial functions, because mitochondria themselves evolved from a gram-negative alpha-proteobacterium, which *E. coli* also falls under (Gray, 2012). The gram-positive *B. thuringiensis* showed little to no sensitivity to *p*-NPIC as well. Like *E. coli*, this result is surprising because several naturally occurring isocyanide products are highly effective against both gram-negative and gram-positive bacteria (Raffa et al., 2020; Zhu et al., 2018). One possible explanation for this result could be that *B. thuringiensis* is an organism that is commonly found in soil (Argôlo-

Filho & Loguercio, 2013), and therefore could have mechanisms that allow it to resist *p*-NPIC. One such mechanism, discussed in chapter 1, could be an isocyanide hydratase. Isocyanide hydratase functions by breaking down the triple bond between the nitrogen and carbon atoms of the isocyanide functional group into an N-substituted formamide (Goda et al., 2001). A few different isocyanide hydratases have been discovered, most notably from other soil organisms, *Pseudomonas putida* (Goda et al., 2001) and *Arthrobacter pascens* (Fukatsu et al., 2004). It is not outside the realm of possibility that the strain of *B. thuringiensis* that was used in this study could have contained an isocyanide hydratase. This would also be an interesting follow-up experiment to this research.

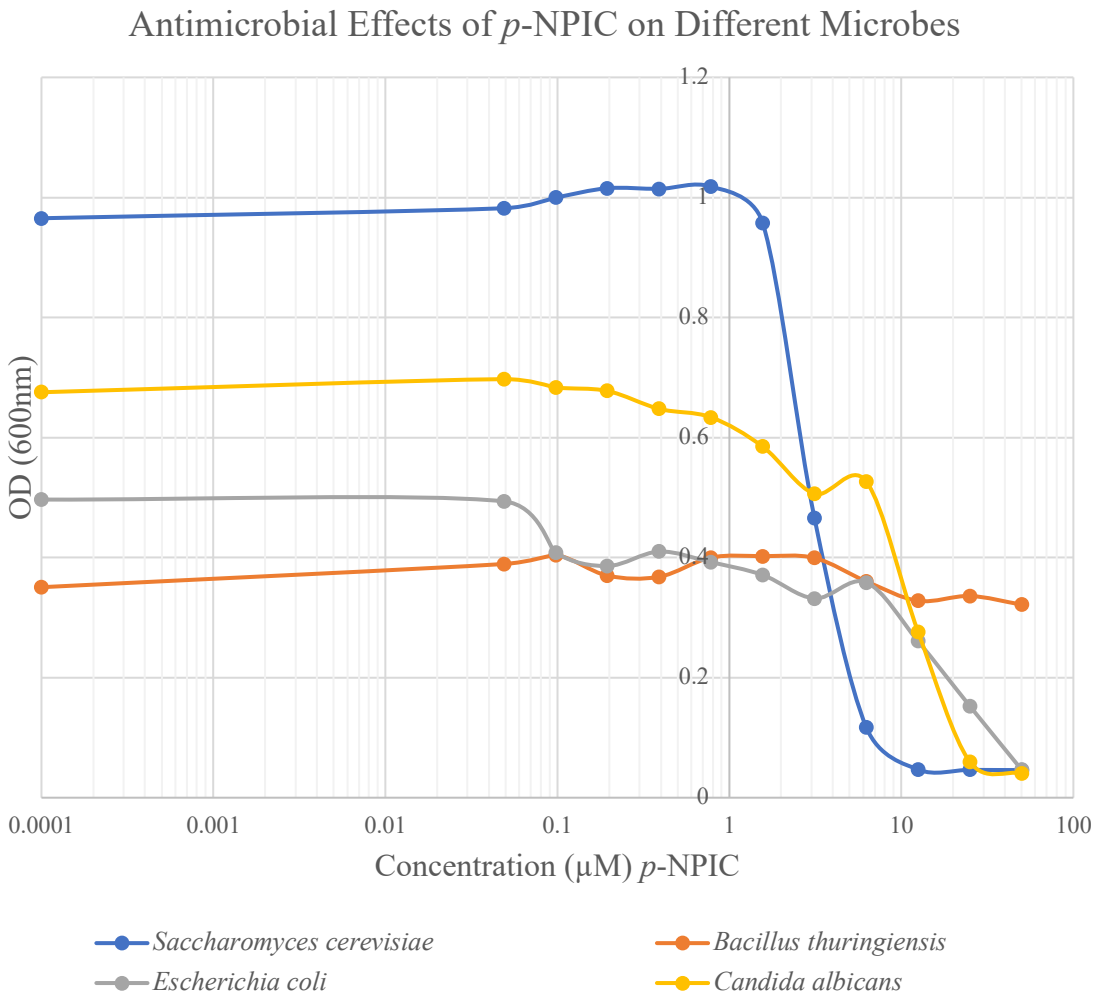

Figure 2.3: Minimum inhibitory concentrations of 4 different types of microbes against *p*-NPIC. The two fungi, *S. cerevisiae* strain BY4741 and *C. albicans* had a much lower MIC values, 10.5 µM and 25 µM respectively, compared to the gram-negative *E. coli* 50 µM and the gram-positive *B. thuringiensis*.

p-nitrophenyl isocyanide shows mixed inhibition effects

After observing the inhibition of growth of fungi with a low micromolar dose of *p*-NPIC, we wanted to see if *p*-NPIC was acting as a fungicide and killing the cells or simply inhibiting their growth for a period of time, being fungistatic. Several isocyanide natural compounds are cytocidal (Schnermann & Shenvi, 2015), killing the organisms they effect, and so we wanted to see if *p*-NPIC would follow a similar trend. To test this, BY4741 *S. cerevisiae* cells were treated with several concentrations of *p*-NPIC (0 μM control, 3 μM, 4.5 μM, and 6 μM) and aliquots of the cells were taken at one hour and 24 hours and plated to test for viable colonies. One hour after the addition of *p*-NPIC, *S. cerevisiae* cells at all three concentrations; 3 μM, 4.5 μM, and 6 μM decreased in viable colony counts from an average of 273.67 colonies on the control to 131.67, 134.33, and 126.5 colonies on average, respectively (**Figure 2.4**). 24 hours after the addition of *p*-NPIC, the viable colony counts of 3 μM, 4.5 μM, and 6 μM, compared to the average 795 colonies from the control, were 196.33, 173, and 178 colonies on average, respectively (**Figure 2.4**).

While there is a decrease in colony counts compared to the control at both one hour and 24 hours, there are still viable colonies that grew. These results imply that, unlike its isocyanide natural product counterparts, *p*-NPIC shows mixed inhibitory effects, with the ability to inhibit ~75% of growth, but not completely stop it. With the preliminary in vitro effects on fungi as shown in **Figures 2.3** and **2.4**, *p*-NPIC has the potential to be tested as an antifungal compound in in vivo studies. With the limited classes of antifungal compounds available, new classes of antifungal drugs are crucial for the treatment of fungal diseases with resistances to the known classes of antifungals, even

if they are fungistatic drugs that only inhibit growth for a certain period of time, like *p*-NPIC. To better understand the mechanisms by which *p*-NPIC acts as a fungistatic compound, the next section will breakdown the results from a genetic screen using *p*-NPIC.

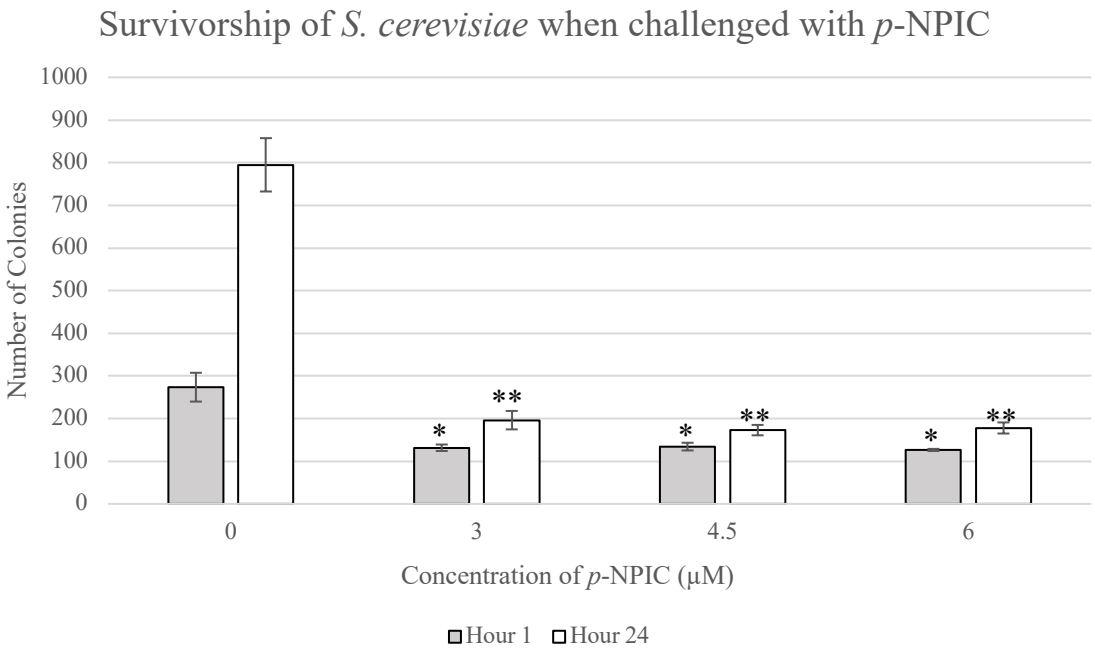

Figure 2.4: Survivorship assay of colonies of *Saccharomyces cerevisiae* strain BY4741 on solid YPD media when challenged with different concentrations of *p*-NPIC. This graph depicts the 10^{-5} dilutions from both the 1 hour and 24 hour plate counts. Each concentration and time point were completed as technical triplicates.

p-nitrophenyl isocyanide effects mitochondrial functions in S. cerevisiae

Of the roughly ~4,800 non-essential gene knockout strains tested, 167 of the mutants were sensitive to *p*-NPIC on both solid and liquid media. A complete list of all gene hits plus their MIC in liquid media can be found in **Table 2.3**. A receiver-operating characteristic (ROC) curve analysis was performed on the hits from the *p*-NPIC genetic screen to determine how related the hits from the genetic screen are. A highly connected set of gene hits implies that there is high similarity between the functionalities among the gene hits. Compared to two other sets of 167 randomly chosen genes from the knockout collection, the 167 genes that were found from our genetic screen were more closely related than the random sets of genes were. The genes pulled from the *p*-NPIC screen had a P-value of 1.327903e-17 and an area under the curve (AUC) value of 0.7075, while random gene sets one and two had AUC values of 0.5405 and 0.532 respectively (**Figure 2.5**). YeastNet defines any gene set that has an AUC value of > 0.7 to be significantly related to other hits from the same genetic screen, compared to random gene sets. Our results imply that the 167 genes found from the genetic screen had connected functionalities in the cell.

After ROC analysis, the *p*-NPIC genetic screen hits underwent genetic ontology (GO) analysis. Of the 167 sensitive mutants identified, 35.9% of mutants were genes that were directly involved with mitochondrial function. The next highest gene class that was represented in the genetic screen was vacuolar membrane ATPase function, with 6% of the gene hits. Another significant source of the gene hits came from genes associated with iron-sulfur and copper binding, making up 4.2% of hits. A summary of the gene classes found from the genetic screen can be found in **Table 2.4**. The entire breakdown of

the genetic screen by gene ontology with their respective P-values can be found in **Table 2.5**.

The most sensitive deletion-strains (MIC < 3.0 μM in liquid media) were enriched in mitochondrial functions including the mitochondrial type II fatty acid synthase, lipoic acid biosynthesis and protein lipoylation, synthesis and assembly of iron-sulfur clusters, and assembly and maintenance of cytochrome C oxidase. The identification of essentially all components of the proton-pumping vacuolar membrane ATPase, as well as some pH sensitive components of membrane lipid biosynthesis, also suggest a role for regulation of cytoplasmic pH as a key determinant of *p*-NPIC tolerance.

Table 2.3: Full list of 167 gene hits from *p*-nitrophenyl isocyanide genetic screen, along with 50% minimum inhibitory concentration (MIC) values (μM) in liquid media.

Gene ID	Name	50% MIC (μM)	Description
YMR072W	ABF2	1.5	Mitochondrial DNA-binding protein
YMR064W	AEP1	2	Protein required for expression of OLI1
YER087W	AIM10	3.5	Protein with similarity to tRNA synthetases
YIL158W	AIM20	1.75	Protein of unknown function
YJL046W	AIM22	1	Putative lipoate-protein ligase
YML087C	AIM33	2	Protein of unknown function
YLR393W	ATP10	2.5	Mitochondrial inner membrane protein
YNL315C	ATP11	0.9	Molecular chaperone
YML081C-A	ATP18	3	Subunit of F1F0 ATP synthase
YDR350C	ATP22	1	Translational activator for ATP6 mRNA
YER061C	CEM1	1	Condensing enzyme w/ mitochondrial function
YLR346C	CIS1	3.5	Protein of unknown function
YLR348C	DIC1	1.5	Mitochondrial dicarboxylate carrier
YBR026C	ETR1	0.9	2-enoyl thioester reductase
YBL013W	FMT1	3.5	Methionyl-tRNA formyltransferase
YPL262W	FUM1	1.7	Fumarase
YAL044C	GCV3	0.7	H subunit of the mitochondrial GDC
YHR067W	HTD2	0.8	3-hydroxyacyl-thioester dehydratase
YDL066W	IDP1	1.75	NADP-specific isocitrate dehydrogenase
YDR148C	KGD2	3.2	Dihydrolipoyl transsuccinylase
YOR221C	MCT1	1.25	Malonyl-CoA:ACP transferase
YLR069C	MEF1	0.9	Mitochondrial elongation factor
YJR077C	MIR1	1.25	Mitochondrial phosphate carrier
YMR166C	MME1	2	MIM magnesium transporter
YLL006W	MMM1	0.4	ER integral membrane protein
YLR190W	MMR1	2.5	Phosphorylated protein of MOM
YGR243W	MPC3	3.5	Subunit of the mitochondrial pyruvate carrier
YNL005C	MRP7	3.5	Mitochondrial ribosomal protein, large subunit
YBL090W	MRP21	3.5	Mitochondrial ribosomal protein, small subunit
YLR439W	MRPL4	1.25	Mitochondrial ribosomal protein, large subunit
YDR237W	MRPL7	3	Mitochondrial ribosomal protein, large subunit
YGR220C	MRPL9	3.25	Mitochondrial ribosomal protein, large subunit
YNL252C	MRPL17	3.5	Mitochondrial ribosomal protein, large subunit
YNL177C	MRPL22	3.5	Mitochondrial ribosomal protein, large subunit
YMR193W	MRPL24	0.7	Mitochondrial ribosomal protein, large subunit
YKL138C	MRPL31	2	Mitochondrial ribosomal protein, large subunit
YDR322W	MRPL35	3.5	Mitochondrial ribosomal protein, large subunit
YBR268W	MRPL37	1.5	Mitochondrial ribosomal protein, large subunit
YKL170W	MRPL38	1.25	Mitochondrial ribosomal protein, large subunit
YPL173W	MRPL40	0.8	Mitochondrial ribosomal protein, large subunit
YIR021W	MRS1	2.25	Splicing protein
YDR115W	MRX14	1	Mitochondrial ribosomal protein, large subunit
YOL033W	MSE1	0.8	Mitochondrial aminoacyl-tRNA synthetase
YHR168W	MTG2	3.5	Putative GTPase
YKL055C	OAR1	0.625	3-oxoacyl-[acyl-carrier-protein] reductase
YKL134C	OCT1	2	Octapeptidyl aminopeptidase
YER178W	PDA1	0.3	E1 alpha subunit of the PDH complex
YBR221C	PDB1	1.8	E1 beta subunit of the PDH complex

YGR193C	PDX1	2.5	E3 binding protein of the PDH complex
YMR267W	PPA2	1.75	Mitochondrial inorganic pyrophosphatase
YCR079W	PTC6	1.5	Mitochondrial type 2C protein phosphatase
YBL057C	PTH2	2	Mitochondrial peptidyl-tRNA hydrolases
YCR028C-A	RIM1	3.5	Involved in mitochondrial DNA replication
YEL050C	RML2	1.5	Mitochondrial ribosomal protein, large subunit
YFL036W	RPO41	3.5	Mitochondrial RNA polymerase
YPR116W	RRG8	1.75	Protein of unknown function
YER050C	RSM18	1.75	Mitochondrial ribosomal protein, small subunit
YKL155C	RSM22	1.75	Mitochondrial ribosomal protein, small subunit
YGR215W	RSM27	1.25	Mitochondrial ribosomal protein, small subunit
YLR139C	SLS1	1.75	Mitochondrial membrane protein
YPR024W	YME1	1.75	Catalytic subunit of i-AAA protease complex
YKL220C	FRE2	3	Ferric reductase and cupric reductase
YER145C	FTR1	3	High affinity iron permease
YPL059W	GRX5	2.5	Glutathione-dependent oxidoreductase
YJR122W	IBA57	3	Involved with iron-sulfur clusters
YKL040C	NFU1	1.25	Fe-S cluster transfer to mitochondrial clients
YGL167C	PMR1	2.5	High affinity Ca2+/Mn2+ P-type ATPase
YBR037C	SCO1	3.5	Copper-binding protein of MIM
YOR078W	BUD21	3	Component of small SSU processosome
YDR382W	RPP2B	3.5	Ribosomal protein P2 beta
YPR132W	RPS23B	3	rp28 of the small ribosomal subunit
YER131W	RPS26B	3.5	Mitochondrial ribosomal protein, small subunit
YDR197W	CBS2	1.5	Translational activator of the COB mRNA
YMR256C	COX7	0.625	Subunit VII of cytochrome c oxidase
YLL018C-A	COX19	2.5	Required for cytochrome c oxidase assembly
YER154W	OXA1	0.4	MIM insertase
YPR191W	QCR2	1.75	Subunit 2 of ubiquinol cytochrome-c reductase
YLR192C	HCR1	1.75	eIF3j component of eIF3
YPL184C	MRN1	3	Translational regulation RNA-binding protein
YPR163C	TIF3	1.5	Translation initiation factor eIF-4B
YEL027W	VMA3	2.5	Subunit c of V0 of vacuolar H(+)-ATPase
YOR332W	VMA4	1.8	Subunit E of V1 domain of the V-ATPase
YLR447C	VMA6	0.9	Subunit D of V0 IM domain of V-ATPase
YGR020C	VMA7	1.75	Subunit F of V1 PM domain of V-ATPase
YEL051W	VMA8	2.5	Subunit D of V1 PM domain of V-ATPase
YHR039C-B	VMA10	1.25	Subunit G of V1 PM domain of V-ATPase
YPL234C	VMA11	1.8	Subunit C of V0 V-ATPase
YPR036W	VMA13	2	Subunit H of V1 PM domain of V-ATPase
YHR026W	VMA16	1.25	Subunit C of the V-ATPase
YKL119C	VPH2	3.25	IM protein required for V-ATPase function
YLR114C	AVL9	1.25	Involved in exocytic transport from the Golgi
YDL099W	BUG1	0.8	Cis-golgi localized protein
YIL041W	GVP36	4.5	Localizes to early and late Golgi vesicles
YJL186W	MNN5	3.5	Alpha-1,2-mannosyltransferase
YER151C	UBP3	1.8	Ubiquitin-specific protease
YLR144C	ACF2	3.5	Intracellular beta-1,3-endoglucanase
YHR093W	AHT1	3.5	Protein of unknown function
YOR067C	ALG8	3.5	Glucosyl transferase
YNL270C	ALP1	3.5	Arginine transporter
YLR089C	ALT1	3.5	Alanine/glutamic pyruvic transaminase
YLL042C	ATG10	0.5	Conserved E2-like conjugating enzyme
YOL083W	ATG34	1.5	Protein involved in selective autophagy
YIL015W	BAR1	3.5	Aspartyl protease
YDR375C	BCS1	3.5	Chaperone required for Complex III assembly

YBR200W	BEM1	3	Involved in establishing cell polarity
YER114C	BOI2	1.75	Protein implicated in polar growth
YNL305C	BXI1	3.5	Protein involved in apoptosis
YAL021C	CCR4	1.5	Component of CCR4-NOT complex
YGL206C	CHC1	1.25	Involved in intracellular protein transport
YCL007C	CWH36	1.75	Dubious open reading frame
YIL029C	EMA17	3.5	Protein of unknown function
YDR512C	EMI1	1.75	Protein of unknown function
YMR015C	ERG5	0.4	C-22 sterol desaturase
YBR041W	FAT1	3.5	Long chain fatty acyl-CoA synthetase
YER027C	GAL83	3.5	beta-subunit of the Snf1 kinase complex
YOR375C	GDH1	1.9	NADP(+)-dependent glutamate dehydrogenase
YLR091W	GEP5	1.5	Protein of unknown function
YJL184W	GON7	3.5	Component of the EKC/KEOPS complex
YOR358W	HAP5	3	Subunit of Hap2p/3p/4p/5p CCAAT complex
YGL251C	HFM1	0.15	Meiosis specific DNA helicase
YDR174W	HMO1	1.75	Chromatin associated HMG family member
YGL168W	HUR1	1.75	Protein of unknown function
YJL153C	INO1	1.8	Inositol-3-phosphate synthase
YOL081W	IRA2	2	GTPase-activating protein
YDR332W	IRC3	3.5	DS DNA-dependent helicase of DExH/D-box
YLL033W	IRC19	0.7	Protein of unknown function
YNL265C	IST1	3.5	Forms a complex with Did2p
YER110C	KAP123	3	Mediates nuclear import of ribosomal proteins
YLR239C	LIP2	0.9	Lipoyl ligase
YNL307C	MCK1	2	ser/thr and tyrosine protein kinase
YGL197W	MDS3	1.75	Component of the TOR regulatory pathway
YNR059W	MNT4	3	alpha-1,3-mannosyltransferase
YMR070W	MOT3	0.8	Transcriptional repressor, activator
YLR219W	MSC3	3.5	Protein of unknown function
YKR082W	NUP133	0.9	Subunit of Nup84p subcomplex of NPC
YOR269W	PAC1	3.5	Component of dynein/dynactin pathway
YBL080C	PET112	3.5	Subunit of GatFAB AmidoTransferase(AdT)
YLR067C	PET309	1.5	Translational activator for the COX1 mRNA
YMR205C	PFK2	0.4	ß-subunit of heterooctameric phosphofructokinase
YIL122W	POG1	1.25	DNA-binding transcriptional activator
YBL046W	PSY4	0.25	Regulatory subunit of protein phosphatase PP4
YBR043C	QDR3	3.5	Multidrug transporter of the facilitator superfamily
YJL217W	REE1	3.5	Involved in the regulation of enolase
YDR195W	REF2	1.25	RNA-binding protein
YLL002W	RTT109	3.5	Histone acetyltransferase
YDR129C	SAC6	2.5	Fimbrin, actin-bundling protein
YOR035C	SHE4	2	Protein containing a UCS domain
YLR079W	SIC1	2.5	Cyclin-dependent kinase inhibitor
YBR156C	SLI15	1.5	Subunit of conserved CPC
YAL009W	SPO7	2	Subunit of Nem1p-Spo7p phosphatase holoenzyme
YOL091W	SPO21	1.75	Involved in modifying the meiotic outer plaque
YOR047C	STD1	3.5	Involved in glucose-regulated gene expression
YOR191W	ULS1	3.5	Swi2/Snf2-related translocase
YLR070C	XYL2	1.75	Xylitol dehydrogenase

Table 2.4: Summary of gene classes found from *p*-NPIC genetic screen. A total of 167 gene hits were found from the *p*-NPIC screen.

Gene Class	# of Sensitive Mutants	% of Hits
Mitochondrial Function	60	35.9%
Vacuolar Membrane ATPase	10	6%
Iron-sulfur and Copper Binding	7	4.2%
Cytochrome C Function	5	3%
Ribosomal Function	4	2.4%
Translation Factors	3	1.8%
Other	78	46.7%

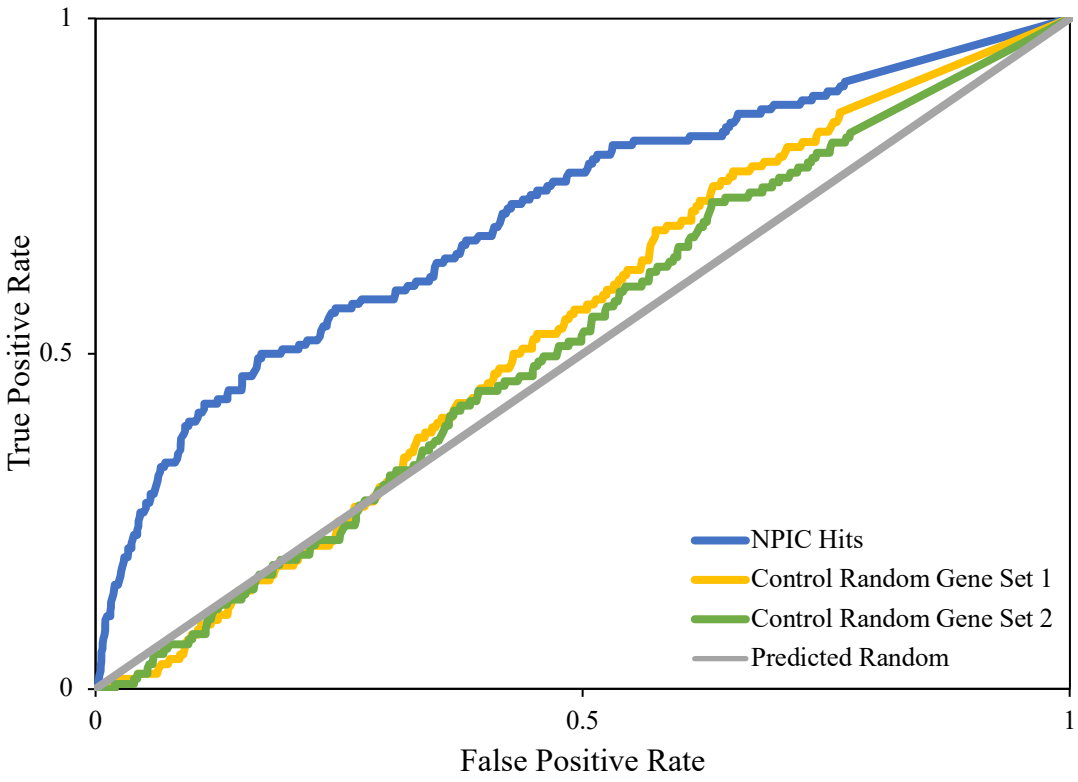

Figure 2.5: Receiver-operating characteristic (ROC) curve analysis from the hits of the *p*-NPIC genetic screen. ROC analysis was done as described in text using the YeastNet v3 (Kim et al., 2014). Two different sets of 167 randomly selected genes were analyzed to serve as a comparison to the 167 gene hits found in the *p*-NPIC genetic screen. The P-value for the ROC analysis was 1.327903e-17. The Area Under the Curve (AUC) value for *p*-NPIC hits was 0.7075 compared to the two control random gene sets, which had AUC values of 0.5405 and 0.5232, respectively.

Table 2.5: Gene Ontology (GO) analysis of the 167 identified hits from the *p*-NPIC genetic screen. P-values were calculated based on the hypergeometric test, any GO terms that have a P-value of < 0.05 are listed in this table.

GO Identifier	GO Component (C) or Process (P)	Identified	P-value
GO:0005739	C: Mitochondrion	71	6.87E-19
GO:0005762	C: Mitochondrial large ribosomal subunit	14	4.626E-13
GO:0033179	C: Proton-transporting V-type ATPase, V0 domain	5	5.676E-09
GO:0042645	C: Mitochondrial nucleoid	8	4.565E-08
GO:0000220	C: Vacuolar proton-transporting V-type ATPase, V0 domain	5	1.149E-07
GO:0000221	C: Vacuolar proton-transporting V-type ATPase, V1 domain	5	3.007E-07
GO:0005840	C: Ribosome	21	4.698E-06
GO:0030529	C: Ribonucleoprotein complex	21	5.206E-06
GO:0005759	C: Mitochondrial matrix	12	8.218E-06
GO:0033177	C: Proton-transporting domain of ATPase complex	4	8.521E-06
GO:0005967	C: Mitochondrial pyruvate dehydrogenase complex	4	0.000045
GO:0005743	C: Mitochondrial inner membrane	3	0.001288
GO:0031235	C: Intrinsic to internal side of plasma membrane	2	0.002994
GO:0030061	C: Mitochondrial crista	2	0.002994
GO:0033178	C: Proton-transporting ATPase complex, catalytic domain	2	0.007265
GO:0015934	C: Large ribosomal subunit	3	0.007326
GO:0005763	C: Mitochondrial small ribosomal subunit	4	0.007778
GO:0005622	C: Intracellular	15	0.009981
GO:0000329	C: Fungal-type vacuole membrane	7	0.04121
GO:0005774	C: Vacuolar membrane	7	0.04676
GO:0007035	P: Vacuolar acidification	11	3.251E-12
GO:0032543	P: Mitochondrial translation	18	2.107E-11
GO:0015992	P: Proton transport	12	7.286E-11
GO:0015991	P: ATP hydrolysis coupled proton transport	8	6.457E-10
GO:0006811	P: Ion transport	14	1.449E-07
GO:0006412	P: Translation	21	5.476E-07
GO:0000002	P: Mitochondrial genome maintenance	8	1.034E-06
GO:0006086	P: Acetyl-CoA biosynthetic process from pyruvate	3	0.000045
GO:0009249	P: Protein lipoylation	3	0.000045
GO:0033615	P: Mitochondrial proton-transporting ATP synthase assembly	4	0.000048
GO:0006633	P: Fatty acid biosynthetic process	5	0.000086
GO:0009060	P: Aerobic respiration	7	0.000277
GO:0006461	P: Protein complex assembly	5	0.000365
GO:0006631	P: Fatty acid metabolic process	5	0.001376
GO:0032979	P: Protein insertion into mitochondrial membrane	2	0.007265
GO:0007533	P: Mating type switching	2	0.01002
GO:0016226	P: Iron-sulfur cluster assembly	3	0.01294
GO:0006096	P: Glycolysis	3	0.02045
GO:0006537	P: Glutamate biosynthetic process	2	0.02923
GO:0006878	P: Cellular copper ion homeostasis	2	0.02923
GO:0000032	P: Cell wall mannoprotein biosynthetic process	2	0.02923
GO:0006099	P: Tricarboxylic acid cycle	3	0.02991
GO:0055114	P: Oxidation-reduction process	12	0.03181
GO:0006825	P: Copper ion transport	2	0.03404
GO:0007033	P: Vacuole organization	2	0.03404
GO:0006629	P: Lipid metabolic process	7	0.04676

MIC differences between two strains of S. cerevisiae

To ensure that the results from our *p*-NPIC genetic screen were due to the gene knockouts themselves, and not individual *S. cerevisiae* strain characteristics, transformation of a few select gene mutants into a new background strain of *S. cerevisiae* needed to occur. MIC values were recorded from the BY4741 background strain, and after transformation, were then recorded in the SEY6210 background strain as well.

The MIC value for the BY4741 background strain was 10.5 μM. The gene mutants in the BY4741 background: *oar*1, *gcv*3, and *nfu*1 had MIC values of 1.5 μM, 1.5 μM, and 3 μM respectively (**Figure 2.6**). The MIC value for the SEY6210 background strain was also 10.5 μM. The transformed gene mutants in the SEY6210 background: *oar*1, *gcv*3, and *nfu*1had MIC values of 1.5 μM, 1.5 μM, and 1.5 μM respectively (**Figure 2.7**). Both background strains BY4741 and SEY6210 as well as gene mutants from both strains *oar*1 and *gcv*3 had the same MIC value. The MIC for the gene mutant *nfu*1 differed 2-fold between BY4741 and SEY6210.

In all *S. cerevisiae* strains there is a four-part transcription factor for mitochondrial development which is encoded by the genes HAP1, HAP2, HAP3, and HAP4. The BY4741 background strain of *S. cerevisiae* is derived from the *S. cerevisiae* strain S288C. In strains that are derived from S288C, including BY4741, there is a transposon insertion on the C-terminus of HAP1. HAP1's function is to communicate the redox state of a mitochondrion to the cell's nucleus. The transposon insertion found in S288C derived strains inhibits the ability of the cell to form fully functional mitochondria and is responsible for an elevated level of petite colony formation. With a high number of mitochondrial hits from our genetic screen it was imperative to ensure that these results

were not an artifact of the transposon insertion in HAP1. The SEY6210 strain is not derived from SC288C, and therefore does not have the same mitochondrial defects, which made it an optimal strain to compare MIC values to.

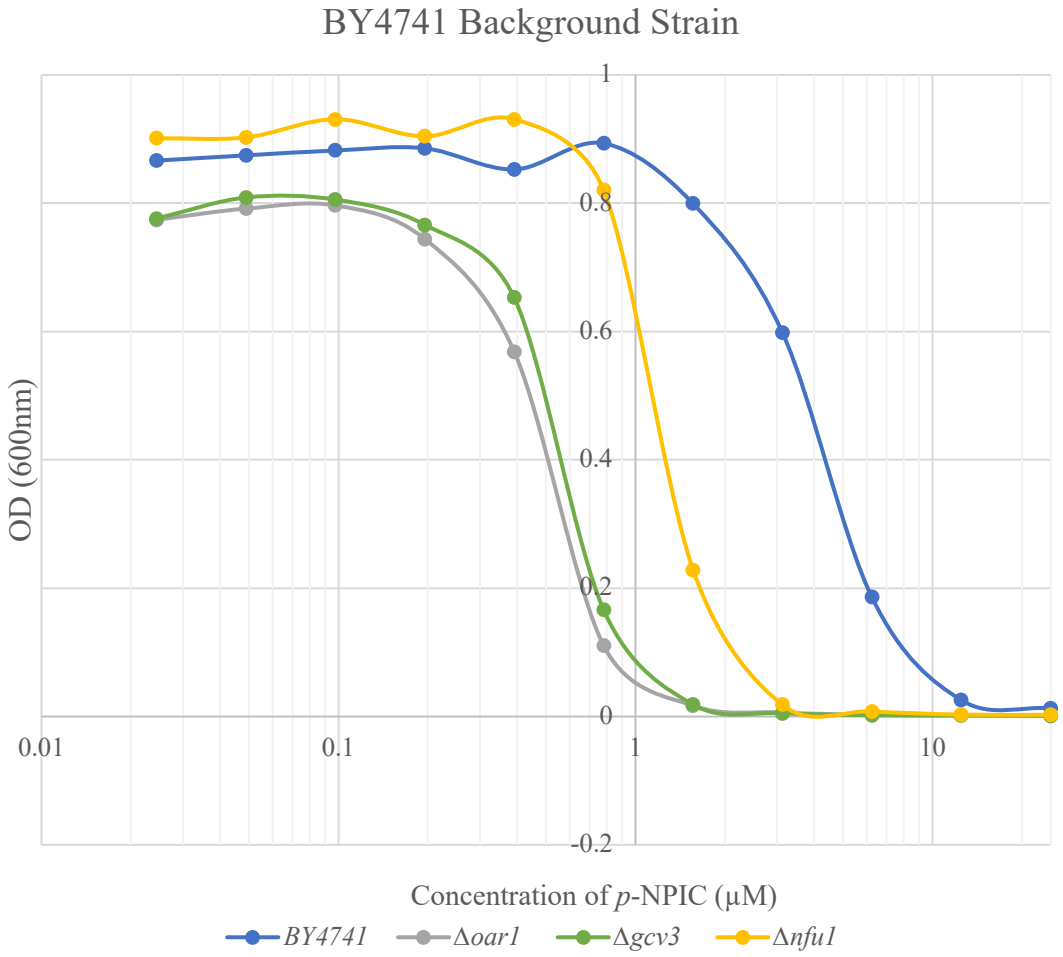

Figure 2.6: Growth curves of background strain BY4741 and mutants *Δgcv3* (part of the mitochondrial glycine decarboxylase), *Δoar1* (mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase), and *Δnfu1* (involved in Fe-S cluster transfer to mitochondrial clients).

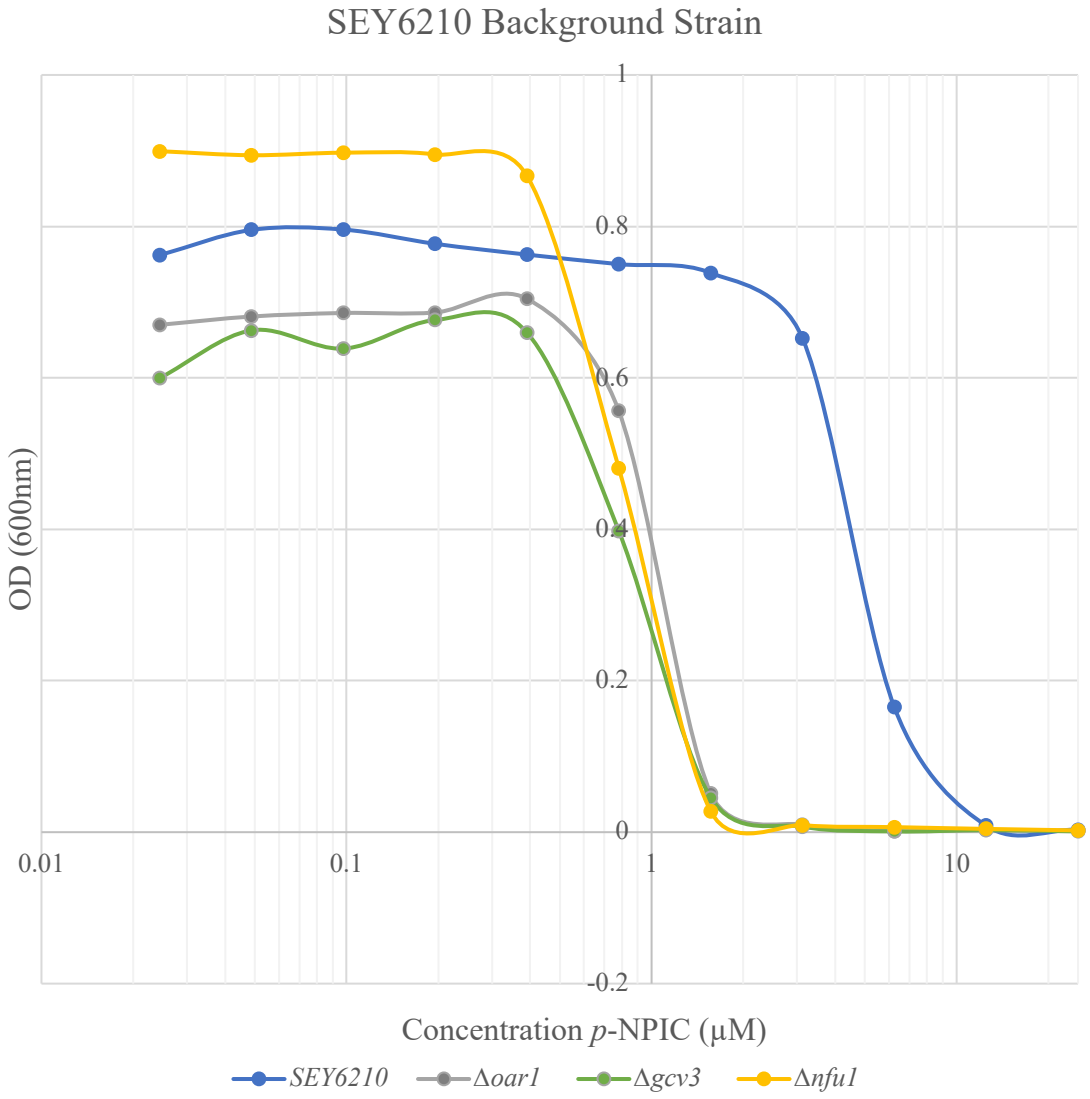

Figure 2.7: Growth curves of background strain SEY6210 and mutants *Δgcv3* (part of the mitochondrial glycine decarboxylase), *Δoar1* (mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase), and *Δnfu1* (involved in Fe-S cluster transfer to mitochondrial clients).

p-nitrophenyl isocyanide acts as a copper chelator

Hits from our genetic screen revealed genes involved with cellular copper ion homeostasis (**Table 2.4**). This result was interesting because several isocyanide natural products have been noted to be copper chelators (Raffa et al., 2020; Zhu et al., 2018). BY4741 *S. cerevisiae* cells were grown in a concentration of 10 μM of *p*-NPIC that, based on previous results, should be too toxic for them to withstand. As can be seen in **Figure 2.8**, the cells grown in 10 μM of *p*-NPIC were not able to grow when no extracellular $CuSO_4$ was added to the media. Around 0.5 mM – 1 mM of $CuSO_4$, we see an increase in the growth of *S. cerevisiae* cells that are challenged with *p*-NPIC, with the OD_{600} peaking at 2 mM of $CuSO_4$. Quickly after the 2 mM concentration of $CuSO_4$, *S. cerevisiae* cell growth tapers off again. At around 2 mM concentration of $CuSO_4$, we suspect that the *p*-NPIC has chelated the intracellular copper and the extracellular copper was able to supplement the copper that *p*-NPIC chelated, allowing for cell growth to occur.

In comparison to the *S. cerevisiae* grown with *p*-NPIC, we also see a spike in growth around the 2mM concentration in cells that are grown without *p*-NPIC (**Figure 2.8**). Liang & Zhou (2007) show that low mM concentrations of $CuSO_4$ enhance cell growth in *S. cerevisiae*. The notable difference between *S. cerevisiae* cells growth without *p*-NPIC versus with 10 μM is shown at the "zero" concentration (noted in this graph as 0.001 for logarithmic purposes). The *S. cerevisiae* cells without *p*-NPIC starts at an OD_{600} of 0.35, while the cells with a toxic concentration (10 μM) start at an OD_{600} of 0.0, which helps confirm that without the aid of extracellular $CuSO_4$ to supplement cell

growth hindered by *p*-NPIC, there would be no cellular growth in the experimental group.

Copper chelation has been a noted mechanism of action of several naturally occurring isocyanide compounds. The isocyanide group has been described as electron rich (Harris et al., 2018). The electronic structure of the isocyanide functional group makes it a prime structure for bonding with transition metals, such as iron and copper (Massarotti et al., 2021), both of which act as metalloenzymes in critical cellular processes such as cellular respiration. In **Figure 2.8** we demonstrate that *p*-NPIC is sequestering intracellular copper, which is then able to be supplemented to restore growth with extracellular copper, which may explain in part how *p*-NPIC is able to act as an antifungal compound.

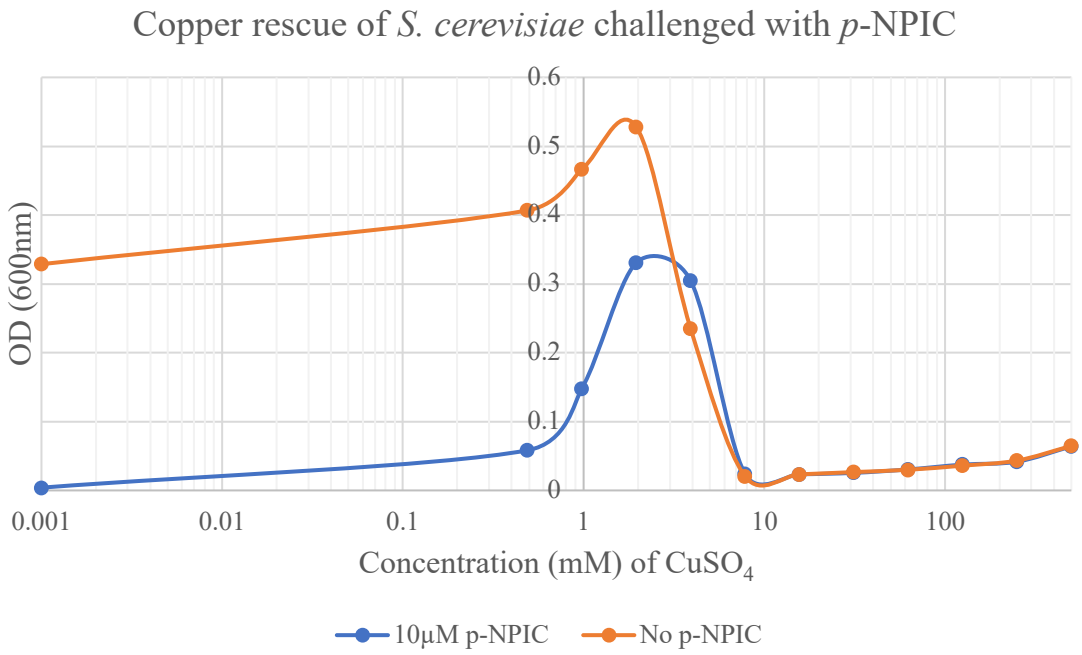

Figure 2.8: Copper rescue of *p*-NPIC challenged *S. cerevisiae* cells. Cells that were challenged with 10 µM of *p*-NPIC were able to grow in 2 mM of $CuSO_4$. Concentrations larger than 2 mM of $CuSO_4$ were too toxic to *S. cerevisiae* cells, while concentrations less than 1 mM of $CuSO_4$ were not concentrated enough to rescue cell growth hindered by *p*-NPIC.

p-nitrophenyl isocyanide increases reactive oxygen species in S. cerevisiae

Since many of the hits from the genetic screen were mitochondrially related, we wanted to see if *p*-NPIC was inducing reactive oxygen species (ROS) formation. ROS can be produced by organelles such as the endoplasmic reticulum, peroxisomes, chloroplasts, and the mitochondria (Nadeem et al., 2013). A previous study on a naturally occurring isocyanide product, SF2768 from *Streptomyces thioluteus* was shown to induce ROS in several species of bacteria (Zhu et al., 2018). We measured relative fluorescence in two strains of *S. cerevisiae*, BY4741 and SEY6210 to compare ROS values between the two. The controls (0 μM) for SEY6210 and BY4741 were 266.89 and 303.19 relative fluorescence units (RFU) respectively (**Figure 2.9**). In the SEY6210 strain, at 2 μM, 4 μM, 6 μM, 8 μM, and 10 μM the RFUs were 589.23, 632.74, 1,267.86, 1,956.77, and 3,388.51 respectively (**Figure 2.9**). In the BY4741 strain at 2 μM, 4 μM, 6 μM, 8 μM, and 10 μM the RFUs were 1,596.49, 601.50, 1,686.27, 1,322.31, and 1,588.24 respectively (**Figure 2.9**). The SEY6210 strain, compared to the BY4741 strain, showed a much clearer stepwise pattern in the increase of ROS in response to an increase in concentration of *p*-NPIC. This could be due to differences in the two background strains. Overall, when compared to the control RFU, both SEY6210 and BY4741 strains showed an increase in levels of ROS.

As stated earlier, ROS can come from several different organelles in the cell. With this assay it is not possible to directly target mitochondrially related ROS individually. This needs to be kept in mind while interpreting the results, the ROS that was measured comes from the entire cell. What these results do confirm is that *p*-NPIC

induces ROS formation in *S. cerevisiae* cells, much like the naturally occurring isocyanide product, SF2768.

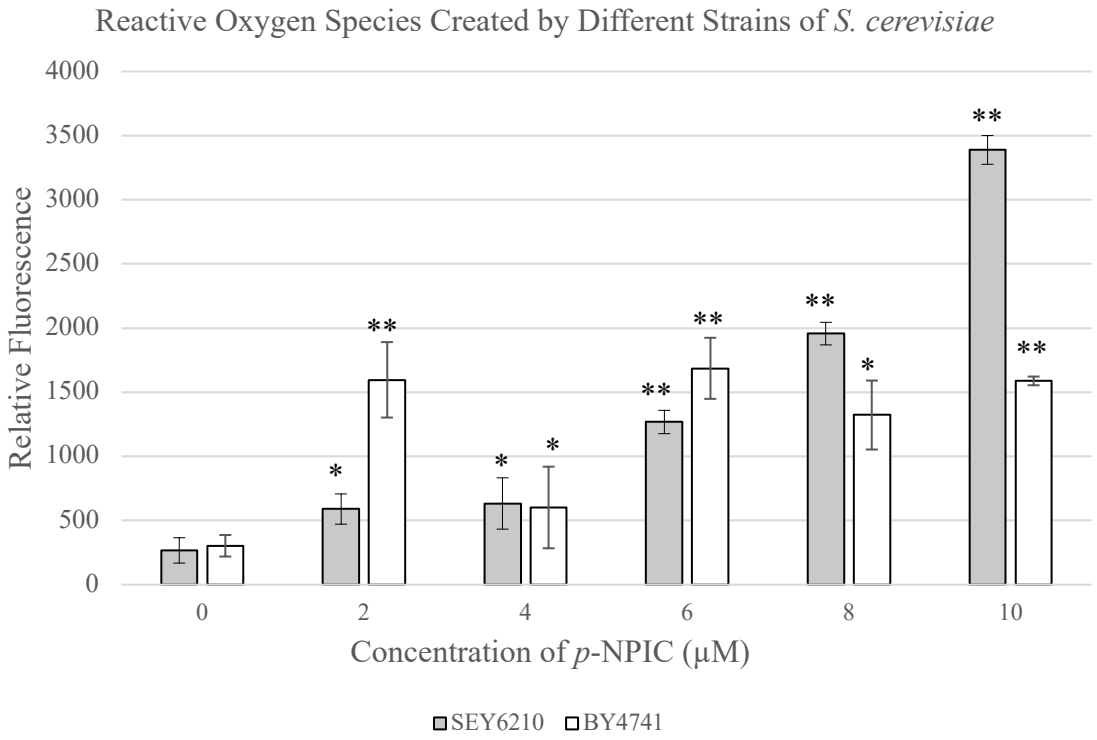

Figure 2.9: Relative fluorescent values indicate an increase of reactive oxygen species (ROS) production for *Saccharomyces cerevisiae* strains BY4741 and SEY6210. Compared to the control, both strains increase in ROS production in the presence of *p*-NPIC. The background strain SEY62120 shows a more stepwise and substantial increase of ROS compared to BY4741.

Conclusions and Future Work

p-NPIC, a synthesized isocyanide compound, is a versatile compound that has great potential in several avenues of research. In this chapter, *p*-NPIC was found to be a potent antifungal compound that targets mitochondrial functions in *Saccharomyces cerevisiae*. This mechanism of action could, in large part, be the result of copper chelating activities and subsequent ROS production by *p*-NPIC. *p*-NPIC shares these qualities with a variety of isocyanide natural compounds (Raffa et al., 2020; Zhu et al., 2018).

These results can be the starting point for a variety of further experimentations. To follow up on the test of the sensitivity of different microbes to *p*-NPIC, it would be worth exploring other species of bacteria, both gram positive and gram negative, to see if they follow the trend seen in our representative microbes. Along with other species of bacteria, it would be of clinical importance to test sensitivity of known human fungal pathogens against *p*-NPIC. Specifically, I would be interested to know the effects that biofilm formation has on the effectivity of *p*-NPIC, since biofilm formation of fungal pathogens is common in human health conditions. To follow up on the genetic screen results, it would be beneficial to perform RNASeq analysis on *Saccharomyces cerevisiae*, to see which genes are up and downregulated when introduced to *p*-NPIC. Another interesting aspect that could be further investigated is looking at how *p*-NPIC interacts with other transition metals, such as iron, magnesium, nickel, etc. A previous study by Hübner et al (2021) has found that iron, incorporated as heme, is another transition metal that isocyanide compounds heavily interact with. This fact also reinforces some of the hits we saw from our genetic screen, which dealt with iron-sulfur cluster formation.

Finally, if *p*-NPIC, or isocyanide compounds in general, are ever going to be considered an option as a new class of antifungals, it is important to test the effects of *p*-NPIC on a multicellular organism such as *C. albicans*. With such a wide variety of functions, the future of research on *p*-NPIC and other isocyanide compounds is very bright.

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