THE ROLE OF FORMATE IN COMBATTING OXIDATIVE STRESS

By

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Abstract

As all aerobic organisms are exposed to oxidative stress, they are known to devise intricate mechanisms to counter reactive oxygen species (ROS). Metabolic networks contributing to the production of ketoacids are prominent in alleviating the oxidative burden. When glyoxylate detoxifies ROS, formate is the principal by-product generated. In this study the contribution of formate in enabling the survival of the microbe *Pseudomonas fluorescens* challenged by hydrogen peroxide (H_2O_2) has been elucidated. When grown in the presence of H_2O_2 (stressed culture), the levels of formate were higher in the spent fluid and the soluble cell-free extracts compared to the controls. Formate was subsequently utilized as a reducing factor to produce NADPH and succinate. The former is mediated by formate dehydrogenase (FDH-NADP), whose activity was enhanced in the stressed cells. Fumarate reductase (FRD) that catalyzes the conversion of fumarate into succinate was also markedly increased in the stressed cells. Metabolic adaptation is a pivotal tool in combatting oxidative stress. Formate, a by-product of glyoxylate- mediated detoxification of ROS is recuperated as a potent reductive fuel. It is becoming quite evident that this simple metabolite has other biological roles that have not been fully appreciated.

Keywords: Formate metabolism, *Pseudomonas fluorescens*, ketoacids, glyoxylate, Formate dehydrogenase, Fumarate reductase- formate dependent, Isocitrate lyase.

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Abbreviations

αKG	Alpha ketoglutarate
BN	Blue Native
CSB	Cell storage Buffer
g	gram
GDH	Glycine dehydrogenase
GTA	Glycine Transaminase
GSH	Glutathione
Hr(s)	hour(s)
ICL	Isocitrate lyase
INT^O	Iodonitro Tetrazolium salt (oxidized)
INT^R	Iodonitro Tetrazolium salt (reduced)
L	Liter
mL	milliliter
min	minute
mM	Millimolar
NAD	Nicotinamide adenine dinucleotide (oxidized form)

NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PAGE	Polyacrylamide Gel electrophoresis
PMS^{O}	Phenazine Methosulfate (oxidized)
PMS^{R}	Phenazine Methosulfate (reduced)
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
Sec	seconds
TCA	Tricarboxylic acid
V	Volt

CHAPTER 1: Introduction & Objectives

1.1 Introduction

1.1.1 Oxidative stress

Cellular metabolism underlies the creation of adenosine triphosphate (ATP), the universal metabolic currency of all organisms. Two major processes are utilized by cells to create ATP, substrate level phosphorylation and oxidative phosphorylation (Rodgers and Wilce, 2000). The latter utilizes oxygen to receive the electrons shunted through the tricarboxylic acid cycle as seen in figure 1.1.

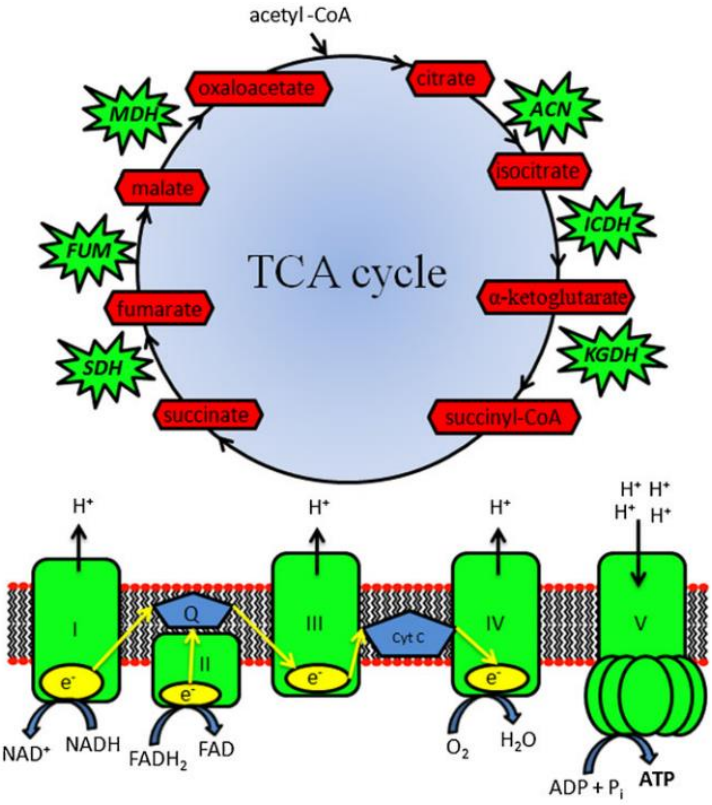

Figure 1.1: Tricarboxylic acid cycle and Electron Transport Chain

The use of oxygen in this ETC primes the system for the creation of oxidative stress. It is estimated that 2-5% of all the O_2 used in the ETC undergoes incomplete reduction by electron leakage which in turn results in higher levels of reactive oxygen species (Cadenas and Davies, 2000). The production of superoxide and hydrogen peroxide occurs in the cytoplasm upon the collision of oxygen with various redox enzymes with solvent exposed flavins (Imlay, 2009). Reactive oxygen species formation can cascade as exemplified in figure 1.2. These ROS may result in metabolic defects, damage to DNA and a variety of other biomolecules (Slauch, 2011).

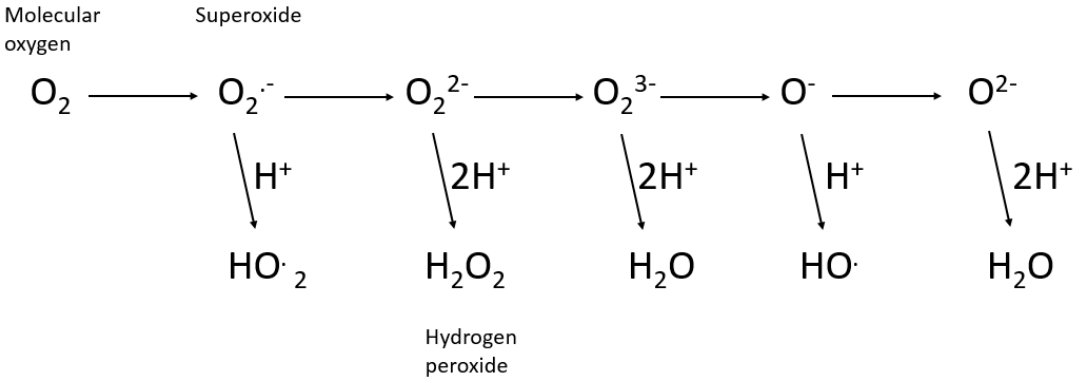

Figure 1.2: Free radical generation from oxygen.

ROS are important components of the antimicrobial defense mechanism incorporated by macrophages and neutrophils (Slauch, 2011). For instance NADPH-dependent phagocytic oxidases (Phox or NOX2) are assembled within the membrane and pump electrons into the compartment to form superoxide anions (O_2^-) which then bombard the invading microbes (Slauch, 2011). As such, several microbes have devised extensive metabolic adaptation strategies to combat

the oxidative stress. By definition oxidative stress simply refers to an imbalance in the pro-oxidant/antioxidant ratio in favor of the former which leads to possible damage (Sies, 1997). Understanding the mechanisms employed by microorganisms to combat this oxidative stress may in the long term provide cues for pharmacologic therapeutic intervention against them. The metabolically versatile *Pseudomonas fluorescens* is a model system for studying some of these adaptation strategies due to its flexible metabolic reconfiguration in the face of stress. It is a gram-negative rod shaped non-pathogenic bacterium that thrives primarily in the soil but on plants and in water surfaces as well (Paulsen et al., 2005) It has simple nutritional requirements and is able to readily grow in mineral media supplemented with a variety of carbon sources (O'sullivan and O'Gara, 1992, Auger et al., 2012).

1.1.2 Detoxification mechanisms

Due to the oxygen-rich atmosphere, microbes are posed with a constant threat of ROS and rely on a vast reserve of enzymes to eliminate the bulk of the stress. Two principal mechanisms govern the maintenance of redox homeostasis; one is predicated on controlling of the redox properties of metalloproteins (NADPH independent) while the other depends on the reduction of NADPH, the latter of which is discussed further in the next section (Mailloux et al., 2010).

The superoxide dismutase family of enzymes catalyse the metal-dependent dismutation of $O_2.^-$ to H_2O_2. Given that the spontaneous reduction of superoxide is unfavorable, the enzymes use two steps to drive the reaction. In the first, SOD accelerates the reduction of the radical anion by

donation of a proton and in the energy generated is utilized to drive the second step with a net result of O_2 and H_2O_2 as seen in figure 1.3 (Wang et al., 2006).

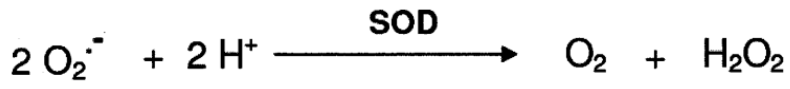

Figure 1.3: Detoxification of the superoxide anion by SOD.

Otherwise, some organisms such as *Desulfovibrio baarsi, Acheoglobus fulgidus* and *Treponema pallidum* utilize superoxide reductase (SOR), a small metalloprotein with an iron-containing active site. They detoxify the superoxide anion into hydrogen peroxide as seen in figure 1.4 (Lombard et al., 2000; Niviere and Fontecave, 2004).

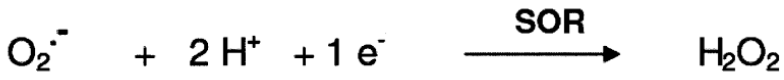

Figure 1.4: Detoxification of superoxide anion using SOR.

Albeit SOD and SOR assist organisms in converting superoxide anions into peroxide, further detoxification is required to quell the threat posed by H_2O_2.

Most commonly utilized with this aim are the catalase enzymes. The majority of catalases are formed of 4 subunits of 60-75 kDa (Kirkman and Gaetani, 2006). The dismutation of hydrogen

peroxide is accomplished by utilizing the catalytic power of the iron-containing heme groups (figure 1.5) (Kirkman and Gaetani, 2006, Wang et al., 2006).

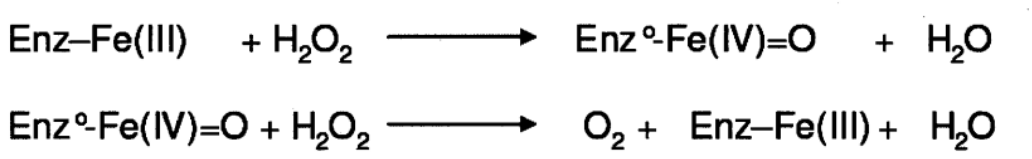

Figure 1.5: Reduction of hydrogen peroxide via the two-step process.

In addition to catalases, thiol-dependent peroxidases also assist in the detoxification of peroxide. These contain small non-heme proteins which catalyse the single electron reduction of peroxides into their corresponding alcohols and depend on their reactive cysteine in the active site (Dubbs and Mongkolsuk, 2007; Poole, 2003). Glutathione peroxidases which are the best characterized peroxidases, mediate the rapid reduction of hydrogen peroxide via the use of a covalently-incorporated selenium in the active site (figure 1.6) (Espinoza et al., 2010; Ursini et al., 1995).

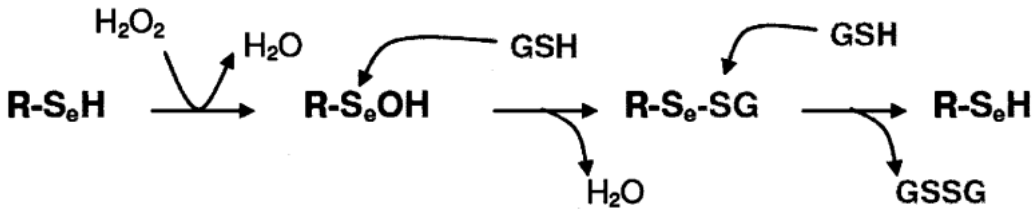

Figure 1.6: Detoxification of hydrogen peroxide via glutathione peroxidase.

In addition to the enzyme-based antioxidative defenses set up by numerous organisms, several incorporate a spectrum of low molecular weight molecules (called low molecular weight antioxidants (LMWA)) which they utilize to scavenge ROS (Grune et al., 2005).

One such moiety is vitamin C, also known as ascorbic acid (Figure 1.7), which is a water-soluble vitamin that reacts with free radicals leading to the generation of an unstable ascorbyl radical which can subsequently be recycled by NAD(P)H- or Glutatione (GSH)-mediated enzymatic activities (Grune et al., 2005; Shao et al., 2008).

Another important LMWA is vitamin E which is a lipid-soluble chain-breaking antioxidant which functions by dividing the compounds into tocopherols or tocotrienols (Figure 1.7). These are lipophilic antioxidants which interact with the polyunsaturated acyl groups of lipids, thereby stabilizing membranes. They scavenge ROS as well as the lipid soluble byproducts of OS (Grune et al., 2005).

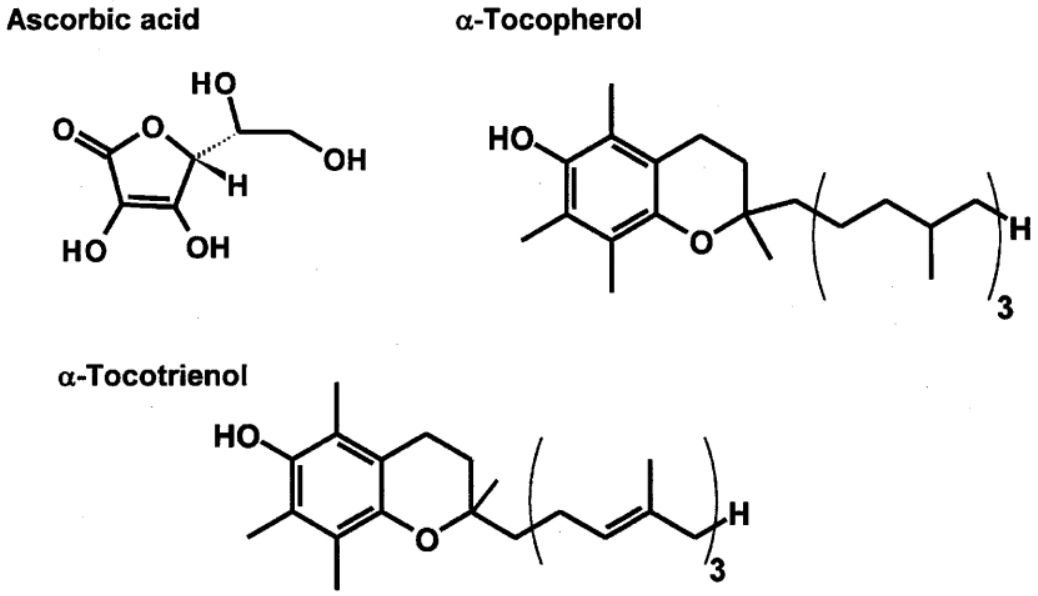

Figure 1.7: Structures of vitamin E and Ascorbic acid.

Complex gene regulation controls these detoxification mechanisms and involves genes such as OxyR, AhpC, and SoxRS (D'Autreaux and Toledano, 2007; Dubbs and Mongkolsuk, 2007; Luschak, 2001). The aforementioned mechanisms of ROS detoxification work in tandem to enable cells to thrive even in toxic oxygenated environments. In recent times the role of metabolism and metabolic shifts in combatting oxidative stress has been greatly studied and elucidated (Mailloux et al., 2006; Ralser et al., 2009). By modulating the catabolic and anabolic pathways, organisms are able to produce key metabolites in the fight against ROS as well as limit the production of pro-oxidants by the TCA cycle.

1.1.3 NADPH/NADH ratio

Several ROS-scavenging systems such as glutathione reductase (GR), as well as ascorbate require NADPH to reduce and regenerate their oxidized active sites whereas others such as catalase utilize NADPH in order to avoid the formation of intermediates which hinder the ability of the enzyme to function (Kirkman and Gaetani, 2006; Singh et al., 2007). Since NADPH is a key component of the anti-oxidative defense mechanisms of cells, NADPH-generating enzymes are highly active under oxidative stress. Some classical enzymes which have been extensively studied include glucose-6-phosphate dehydrogenase (G6PDGH), malic enzyme (ME), NADP-dependent isocitrate dehydrogenase (NADP-ICDH) and NADP glutamate dehydrogenase (NADP-GDH). These function to produce NADPH, thereby keeping the cytosolic compartments of the cell in a constantly reduced environment (Mailloux et al., 2010).

In combatting OS, *P. fluorescens* for instance will not only promote the production of NADPH but will concomitantly decrease the formation of nicotinamide adenine dinucleotide hydride (NADH) with the aim to limit ROS emission from the respiratory chain (Singh et al., 2008). Thus the oxidation of NADH via the complexes of the electron transport chain, I, III and IV which also result in intracellular generation of ROS are diminished (Singh et al., 2008). In summary the combat against OS involves a two pronged approach which ultimately results in the increased production of NADPH and the reduction in the production of NADH (Figure 1.8). For *Pseudomonas fluorescens* cultures grown in citrate media with glycine, this ratio is modulated via

a plethora of metabolic changes. The role of ketoacids in this is only recently begun to be unravelled (Lemire et al., 2010).

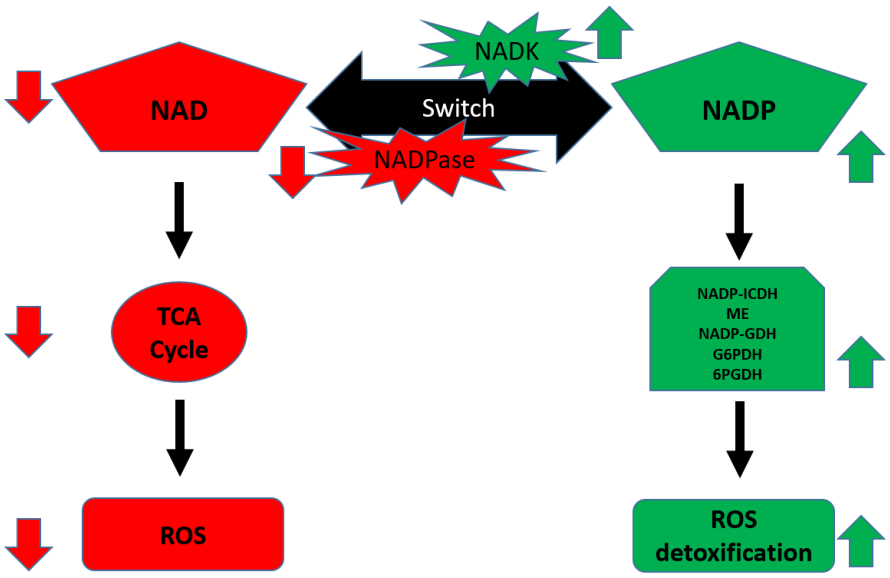

Figure 1.8: The metabolic switch in combatting OS (modified from Mailloux et al., 2010).

1.1.4 Glycine metabolism

Ketoacids react with ROS and concomitantly form their corresponding carboxylic acid as well as CO_2. Pyruvate for example forms acetate by non-enzymatic decarboxylation when neutralizing H_2O_2. Similarly αKG detoxifies ROS and produces succinate in the process (Bignucolo et al., 2013). The production of succinate has the added benefit of fulfilling its role of ketoacids as a potent antioxidant tool (Kohen and Nyska, 2002).

When *Pseudomonas fluorescens* is grown in citrate media exposed to oxidative stress, it shifts their metabolism to produce three key metabolites, ATP, NADPH and glyoxylate to neutralize this threat (Alhasawi et al., 2015). Three key enzymes related to the formation of glyoxylate, Glycine dehydrogenase (GDH), Glycine transaminase (GTA) and Isocitrate lyase (ICL) were found to be upregulated in the oxidatively stressed cells. The production of this ketoacid was discovered to have multiple fates. First was the non-enzymatic decarboxylation into formate and the second was the formation of oxalyl-CoA which is hypothesized to contribute to the formation of ATP. A summary of this is presented in figure 1.9.

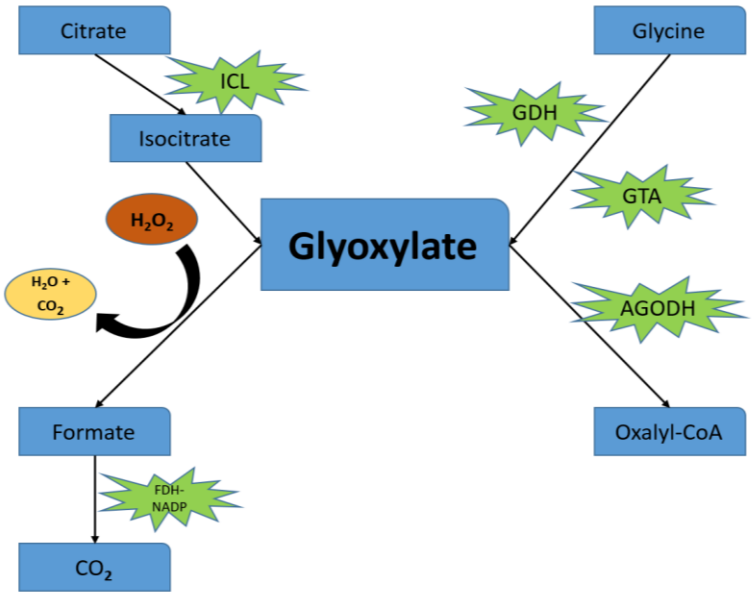

Figure 1.9: Glycine based metabolic shift to combat oxidative stress in *Pseudomonas fluorescens* (Modified from Alhasawi et al., 2015).

The role of the formate generated as consequence of the interaction of glyoxylate with ROS is thought to contribute to NADPH homeostasis. The current study aims at deciphering the contribution of formate in anti-oxidative defense.

1.2 Thesis objectives

The primary objective of this research work is to delineate the role formate plays in *Pseudomonas fluorescens* in combatting oxidative stress. The fate of formate as well as its role in neutralizing oxidative stress can be gauged via a functional proteomic and metabolomics approach using techniques such as blue native polyacrylamide gel electrophoresis (BNPAGE), and high performance electrophoresis (HPLC). The metabolic versatility of *P. fluorescens* makes it an ideal system to evaluate mechanisms involved in fending oxidative stress. In addition to the analysis of antioxidant defense systems, understanding the enzymes mediating the catabolism of various nutritional sources and the metabolites utilized to scavenge ROS will help devise strategies to quell microbes that are resistant to antibiotics possibly by targeting the formate-producing enzymes.

CHAPTER 2: The role of formate in combatting oxidative stress

The role of formate in combatting oxidative stress.

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2.1 Abstract

The interaction of keto-acids with reactive oxygen species (ROS) is known to produce the corresponding carboxylic acid with the concomitant formation of CO_2. Formate is liberated when the keto-acid glyoxylate neutralizes ROS. Here we report on how formate is involved in combating oxidative stress in the nutritionally-versatile *Pseudomonas fluorescens*. When the microbe was subjected to hydrogen peroxide (H_2O_2), the levels of formate were 8 and 2-fold higher in the spent fluid and the soluble cell-free extracts obtained in the stressed cultures compared to the controls respectively. Formate was subsequently utilized as a reducing force to generate NADPH and succinate. The former is mediated by formate dehydrogenase (FDH-NADP), whose activity was enhanced in the stressed cells. Fumarate reductase (FRD) that catalyzes the conversion of fumarate into succinate was also markedly increased in the stressed cells. These enzymes were modulated by H_2O_2. While the stressed whole cells produced copious amounts of formate in the presence of glycine, the cell-free extracts synthesized ATP and succinate from formate. Although the exact role of formate in anti-oxidative defense has to await further investigation, the data in this report suggest that this carboxylic acid may be a potent reductive force against oxidative stress.

Keywords: Formate metabolism, *Pseudomonas fluorescens*, ketoacids, glyoxylate, Formate dehydrogenase, Fumarate reductase- formate dependent, Isocitrate lyase.

2.2 Introduction

As oxidative stress is part of aerobic life, most organisms have evolved intricate mechanisms to circumvent this challenge. These strategies include the utilization of enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase which aid in the lowering of oxidative tension during aerobic respiration (Brioukhanov, Netrusov & Eggen, 2006). Recently the significance of ketoacids in combatting reactive oxygen species (ROS) has emerged. The moieties like alpha ketoglutarate (αKG), oxaloacetate (OAA), pyruvate and glyoxylate scavenge ROS with the concomitant formation of their respective carboxylic acid (Alhasawi et al., 2015a; Lemire et al., 2011; Mailloux, Puiseux-Dao & Appanna, 2008; Singh et al., 2008; Li et al, 2010; Thomas et al., 2015). When glyoxylate is utilized as an ROS scavenger, formate is one of the critical by-products formed (Alhasawi et al., 2015b; Yokota, Kawabata & Kitaoka, 1983; Yokota, Komura & Kitaoka, 1985). In numerous bacteria, plants and animals formate is an important metabolite involved in energy metabolism (Hourton-Cabassa et al., 1998; Leonharsberger, Korsa & Bock, 2002). Its favourable redox potential enables this monocarboxylic acid to be oxidized not only through the aerobic respiratory pathways but to serve as an electron donor for the reduction of key metabolites such as fumarate, nitrate and nitrite (Bagramyan, Galstyan & Trchounian, 2000; Jormakka et al., 2002; Shinagawa et al., 2008; Su & Puls, 2004)

Formate may undergo different fates in these bacteria depending on the physiological conditions (Leonhartsburger et al., 2002). It may be secreted, oxidised aerobically, used as a reductant or converted into CO_2 and H_2 via the enzyme formate hydrogen lyase (Bagramyan &

Trchounian, 2003; Yoshida et al., 2005). A key enzyme in the metabolism of formate is formate dehydrogenase (FDH). In some bacteria this enzyme predominantly utilizes nitrate as an electron acceptor (Uchimura et al., 2002). FDH plays a pivotal role in respiration as well as in the maintenance of a reducing environment (Jormakka, Byrne & Iwata, 2002). These enzymes have been reported with differing cofactor requirements, electron acceptors, substrates and cellular locations (Hourton Cabassa et al., 1998). The FDHs NAD-dependent which have been extensively studied in both bacteria, yeast and plants, have been widely utilized in industry for NADH regeneration (Alekseeva, Savin & Tishkov, 2011; Hoelsch et al., 2013; Suzuki et al., 1998).The occurrence of FDH NADP-dependent has also been reported (Yamamoto et al., 1983; Gul-Karaguler et al., 2001). FDH synthesis has been shown to increase strongly under conditions of stress including abrupt changes in temperature, irradiation with UV light, hypoxia and chemical agents and may contribute in maintaining NADPH homeostasis (Abdreadeku et al., 2009; Andreadeli et al., 2009; Hoelsch et al., 2013; Hourtan Cabassa et al., 1998).

The role of this monocarboxylic acid in providing the reducing power to the pivotal ribonucleotide reductase has been shown (Stubbe et al., 2003). This enzyme participates in the synthesis of deoxyribonucleotides with the concomitant formation of CO_2. Deoxyribonucleotides are critical in the cellular replication and their formation usually necessitates the utilization of NADPH. As part of our study to unravel the significance of metabolic pathways in anti-oxidative defense, we have evaluated the role of formate in the adaptation of the nutritionally-versatile microbe *Pseudomonas fluorescens* to oxidative stress. Here we report that the presence of the

enhanced level of this mono- carbon carboxylic acid is an important source of reducing power in the H_2O_2-challenged cells. It participates in the synthesis of NADPH and contributes to the reduction of fumarate to succinate, biological reactions that ensure the survival of the microbe in an oxidative milieu. The importance of formate as a substitute for NADH in environments where the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OP) are ineffective is also discussed.

2.3 Methods

2.3.1 Growth of *Pseudomonas fluorescens*

Pseudomonas fluorescens from American type culture collection (ATCC13525) was grown in defined citrate/glycine media (control), consisting of Na_2HPO_4 (6g), KH_2PO_4 (3g), 15 mM glycine (1.2g), $MgSO_4.7H_2O$ (0.2g), and 19 mM citrate (4g) with a pH of 6.8. Additionally, trace elements were added as described in (Mailloux et al., 2008). Media were dispensed into 200 mL aliquots in two 500 mL Erlenmeyer flasks (control and stress conditions) and autoclaved for 20 minutes at 121 °C prior to the inoculation with 1ml of bacteria grown to stationary phase in a control medium (same conditions as control culture from the experiment). Oxidative stress in the stressed culture was introduced by the addition of final concentration of 500 µM H_2O_2, an amount known to elicit maximal antioxidative response (Alhasawi et al., 2015a,b). All cultures were aerated in a gyratory water bath shaker, model 76 (New Brunswick Scientific) at 26 °C at 140 rpm. The cells and spent fluid were isolated at the stationary phase of growth for metabolomic and enzymatic analyses (28h for control and 50h growth for the H_2O_2 stressed cultures). Following the harvesting of cells at various growth intervals, the bacterial pellets were treated with 0.5 N NaOH and cell growth was monitored by measuring the solubilized protein using the Bradford assay (Bradford 1976).

2.3.2 Regulation and whole cell experiments

In the regulation experiments, to assess the adaptive and reversible nature of the shifts in metabolism, cells were harvested at stationary phase of growth. Control cells (10 mg protein equivalent) were incubated for 8 hours in 50 mL media containing 500µM H_2O_2 whereas the H_2O_2-stressed cells were incubated for 8 hours in 50 mL control media as described in (Alhasawi et al., 2014). The cell-free extracts and the spent fluid were subsequently analyzed for metabolites and enzymatic activities.

To evaluate the source of formate production, whole cells (10 mg protein equivalent) from the control and stress cultures were incubated for 8 hours in separate media (50 mL) containing the same growth nutrients as the control culture but with only citrate or glycine in the presence and in the absence of H_2O_2. To monitor the rate of formate utilization, 10 mg protein equivalent of control and stressed whole cells were incubated in reaction mixture containing 5 mM formate and the consumption of the monocarboxylic acid was recorded by HPLC after 8 hours.

2.3.3 Cell fractionation

Following the isolation of the bacteria at 4 °C for 10 minutes at 10,000 g with the aid of a Sorvall Legend RT Centrifuge, cells were washed with 0.85% NaCl and respun before being resuspended in 500µL cell storage buffer (CSB) consisting of 50mM Tris-HCl, 5mM $MgCl_2$ and 1mM fluoride (PMSF). Sonication was utilized to lyse the cells (unbroken cells were removed by centrifugation at 10,000g). These were centrifuged at 180,000 g for 3h at 4 °C yielding a soluble

cell-free (CFE) and a membrane fraction. The membrane fraction was suspended in 500µL of CSB. The Bradford assay was utilized to determine protein content with serum bovine albumin as the standard. Equal protein concentrations were utilized in all experiments.

2.3.4 Enzymatic studies

BN-PAGE was executed as per the protocol described in, Auger et al. (2015); Mailloux et al. (2008) and Schagger and von Jagow (1991). For these assays, a 4-16% gradient gel was prepared and the protein (4 µg/µL) was prepared in blue native buffer (400 mM 6-amino hexanoic acid, 50 mM Bis–Tris [pH 7.0]). To solubilize membrane bound proteins in order to ensure optimal protein separation, a final concentration of 1% dodecyl-β-maltoside was added to the membrane fractions. Protein samples were loaded into each well of the native gel (10-60 µg) and electrophoresed at 4 °C under native conditions at 80V and 15mA for proper stacking followed by 150 V and 25 mA in the resolving gel for the migration of the protein until it travelled half-way through the gel. At the halfway point, blue cathode buffer (50 mM Tricine, 15 mM Bis–Tris, 0.02% w/v Coomassie G-250, pH 7 at 4 °C) was changed to a colorless cathode buffer (50 mM Tricine, 15 mM Bis–Tris, pH 7 at 4 °C) to provide improved detection of the protein bands and thence electrophoresis was performed at 300 V and 25 mA. For 15 minutes following the electrophoresis, the gel was incubated in reaction buffer (25 mM Tris–HCl, 5 mM $MgCl_2$ [pH 7.4]), after which, the in-gel activity assay was performed by using a reaction mixture containing equilibrium buffer, 5 mM substrate, 0.5 mM cofactors, 0.2 mg/mL phenazine methosulfate (PMS) or

dichloroindophenol (DCIP), and 0.5 μg/mL iodonitrotetrazolium (INT) in a total volume of 3 mL. For FDH-NADP, this consisted of 5mM formate and 0.5mM NADP whereas the FDH-NAD utilized 0.5mM NAD instead. To confirm the presence of these enzymes the activity bands were cut and incubated in 1 mL reaction mixtures containing the corresponding substrates bicarbonate and NADPH and NADH respectively while monitoring the formation of formate via HPLCs. Fumarate reductase was detected using 5mM fumarate and 0.5mM formate. Confirmation was obtained by incubating the excised bands in a mixture containing fumarate (5mM) and formate (0.5mM) and monitoring for succinate production. Isocitrate lyase, Complex I and NADH were monitored as described in (Auger et al., 2015). Destaining solution (40% methanol and 10% glacial acetic acid) was used to stop the reactions where appropriate. Coomassie staining was used to ensure equal protein loading. The specificity in detections was further confirmed by performing in-gel reactions in the absence of a substrate or by the addition of the inhibitor sodium azide (5mM). Densitometry was performed using imageJ for windows.

Spectrophotometric data for NAD-dependent isocitrate dehydrogenase (ICDH-NAD) was obtained by incubating 1 mg (protein equivalent) of membrane fraction from control and H_2O_2-treated cells with 2 mM isocitrate and 0.5 mM NAD for 1 min. For NADP-dependent ICDH, NADP was used instead of NAD. To monitor malate dehydrogenase (MDH), malate and NAD were utilized. A similar reaction was used for malic enzyme (ME) except NAD was replaced with NADP. NADH and NADPH production were monitored at 340 nm over the course of a minute. Negative controls were performed without the substrates or cofactors.

2.3.5 Monitoring metabolite levels

To evaluate the influence of H_2O_2 stress on metabolic networks, select metabolites (formate, succinate and ATP) were analysed by High Performance Liquid Chromatography (HPLC) (Alhasawi et al., 2015b). Briefly, following the harvesting of the cells at various timed intervals, the spent fluid and the soluble cellular fractions (CFE) were analyzed. An Alliance HPLC with C18 reverse-phase column (Synergi Hydro-RP; 4 μm; 250×4.6 mm, Phenomenex) and Waters dual absorbance detector were utilized. Mobile phase containing 20 mM KH_2PO_4 (pH 2.9) was used at a flow-rate of 0.2 mL/min at ambient temperature to separate the substrates and products, which were measured at 210 nm and 280nm respectively. Peaks were quantified using the Empower software (Waters Corporation and metabolites were identified by spiking biological samples using known standards). HPLC analyses were performed immediately after the reactions in order to minimize substrate and product degradation. Activity bands were excised from the gel and placed in 1 mL reaction mixture containing 2 mM substrates for 30 min of incubation. To monitor FDH-NAD, the excised bands were incubated in reaction mixture containing bicarbonate and NADH whereas to monitor FDH-NADP, the excised bands were incubated in reaction mixture containing bicarbonate and NADPH and in both cases, formate formation was monitored. For fumarate reductase, the reaction mixture comprised fumarate and formate while monitoring for succinate production. The sample (100 μL) was collected and diluted with 900 μL milli-Q water for HPLC analysis.

2.3.6 Statistical analysis

Data were expressed as means ± standard deviations. Percent change was calculated where appropriate in order to account for individual variation and to provide a better measure of the change in activity. The quantitative data were checked for significance using the student t-test ($p \leq 0.05$). All experiments were performed in at least biological duplicates and repeated thrice each.

2.4 Results and Discussion

When subjected to 500 µM H_2O_2, *Pseudomonas fluorescens* produced more formate in the growth media compared to the control cells at stationary phase (Figure 1A). This monocarboxylic acid is known to be a product of the detoxification of ROS by glyoxylate (Alhasawi et al., 2015b). Indeed, formate levels within the soluble cell free extract were also found to be significantly higher in the stressed cells compared to the control cells (Figure 1B). Additionally, when whole cells were incubated with either glycine or citrate for 8 hours, a sharp increase in formate levels was observed compared to the controls (Figure 1C). The activity of isocitrate lyase, an enzyme known to produce glyoxylate was also found to be elevated in the stressed cultures (Figure 1D) (Hamel et al., 2004). As formate was an important product in the cells challenged by H_2O_2, it was important to evaluate how this metabolite was utilized. The stressed whole cells incubated in reaction mixture containing formate consumed this carboxylic acid at a faster rate compared to the control whole cells (data not shown).

As formate was a key metabolite in the stressed cells, it was important to evaluate how this moiety was contributing to the anti-oxidative defense strategy of the microbe. There was a marked increase in the activity of FDH-NADP in the stressed cells (Figure 2A). In an effort to ascertain if this enzyme was being expressed as a consequence of oxidative stress, control cells were exposed to H_2O_2 medium while H_2O_2 challenged cells were incubated in a control medium. A marked reduction of FDH-NADP activity band was observed in the latter while in the former situation the activity band corresponding to the dehydrogenase was enhanced (Figure 2B). FDH-NADP was

readily inhibited by sodium azide (Figure 2C). This enzyme may help contribute to the NADPH budget which is critical in combatting oxidative stress, as both malic enzyme and ICDH-NADP that are known to synthesize this reducing agent were also increased (Table 1) (Beriault et al., 2005; Beriault et al., 2007; Ying, 2008). The stressed cells were also characterized by an increase in FDH-NAD (Figure 2D) which was confirmed by excision of the activity band and incubation in reaction mixture containing NADH and bicarbonate to monitor formate production (Figure 2E). Furthermore this trend was reversed in the regulation experiment as observed (Figure 2F). However, the presence of H_2O_2 resulted in the diminution of the activities of TCA cycle enzymes like ICDH-NAD and MDH (Table 1). Also, Complex I was marked diminished in the stressed cultures (Figure 2G) while the activity band indicative of NADH oxidase was barely evident in the control cells (Figure 2H).

Fumarate reductase (FRD) mediates the conversion of fumarate into succinate with concomitant oxidation of NADH (Appanna et al., 2014). Although this enzyme was present, it readily utilized formate as the reducing cofactor (Figure 3A). Formate is known to provide electrons with the liberation of CO_2 (Zaunmuller et al., 2006). There was a drastic increase in formate dependent FRD in the stressed cells compared to the control cells where the activity band was only slightly visible (Figure 3A). Regulation experiments confirmed the reversible nature of this enzyme (Figure 3B). Furthermore, the formate dependent FRD was distinguished from the NAD-dependent FRD which also showed a marked increase in stress cells (Figure 3C). Incubation of the excised activity band of formate dependent FRD in fumarate and formate yielded succinate

(Figure 3D). The membrane fraction from the stressed cells incubated in reaction mixture containing fumarate, formate and ADP for 30 minutes generated more ATP and succinate compared to the control cells (Figure 3E).

The data in this article point to the ability of formate to act as an important reducing factor in *Pseudomonas fluorescens* exposed to oxidative stress. This carboxylic acid that is referred to as reduced carbon dioxide is known to provide the reducing fuel in a variety of biochemical reactions in lieu of NADH and NADPH. Reduction of ribonucleotide, nitrite and cytochrome C has been shown to be mediated by formate (Stubbe et al., 2003). In this study, formate contributes to the anti-oxidative defense strategy by supplying NADPH. Although a variety of mechanisms are deployed by microorganisms including the synthesis of exopolysaccharides (Appanna & Preston, 1987) to quell oxidative tension, there is a dearth of information on such a role for this carboxylic acid. This metabolite aided by the enzymes ME and ICDH-NADP, may allow *P. fluorescens* to battle the oxidative challenge posed by H_2O_2. Indeed, various organisms are shown to evoke intricate NADPH-generating pathways to modulate their NADPH budget to combat oxidative stress (Alhasawi et al., 2014; Chenier et al., 2008; Drummond et al., 2011; Spanier et al., 2009). The ability of formate to reduce fumarate to succinate with the aid of FRD may prove an added benefit to this microbe as the production of NADH is markedly diminished under oxidative tension (Mailloux et al., 2010). The TCA cycle, a key generator of the catabolic reducing agent, is severely impeded (Mailloux et al., 2007). Additionally, oxidative phosphorylation is downregulated as revealed by the diminished activity of Complex I, a situation that may impede the generation of

NAD. To rectify such an occurrence, the microbe invokes the participation of NADH oxidase, an enzyme whose activity is known to be increased during environmental stress (Chenier et al., 2008). Although FDH-NAD may help alleviate the diminished NADH production in the H_2O_2 medium, the utilization of formate in NADH-requiring processes like in the reduction of fumarate to succinate will be an added benefit during oxidative stress. Hence, reactions necessitating NADH may switch to formate as a reducing factor. The enhanced synthesis of glyoxylate fueled by the increased activity of ICL may argue for such a possibility. It is quite likely that *Pseudomonas fluorescens* may have adopted this strategy. Formate, a by-product of the detoxification of ROS by glyoxylate, may have aptly been utilized by this microbe as a potent reductive power.

Although further molecular studies are required to confirm the significance of formate in anti-oxidative defense, the findings in this study argue for the possibility that *Pseudomonas fluorescens* may invoke the participation of this carboxylic acid in fending the challenge posed by H_2O_2. Formate does not only help generate NADPH but also contributes to synthesize key metabolites like succinate that ensures the survival of the microbe. Hence, metabolic reconfiguration appears to be essential to the adaptation of any organism to changing environmental conditions and in this instance, an apparent by-product is recuperated to contribute to the anti-oxidative defense effort (Figure 4).

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2.6 Figures, legends and table

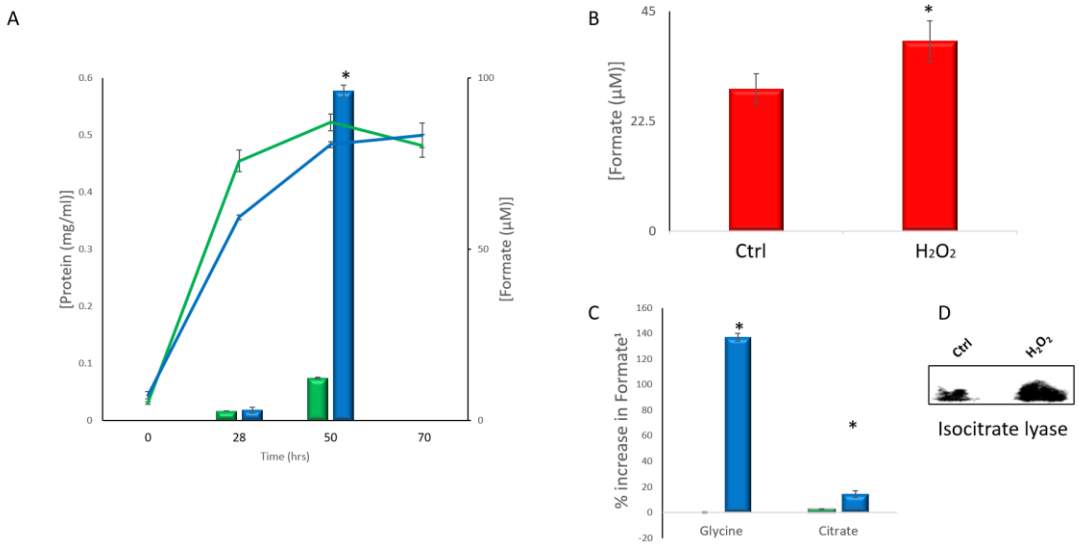

Figure 2.1: Formate production in *P. fluorescens* (A.) Bacterial cell growth as measured by the Bradford assay (protein (mg/ml of culture) and formate production (monitored (by HPLC) in the growth media at various time intervals (control, 500 µM H_2O_2). (B.) Formate levels within the soluble cell free extract (CFE). (C.) Percent increase in formate in a whole cell experiment in which cells were taken at stationary phase from control & 500µM H_2O_2 (stress) and re-suspended in control and stress media (control, 500µM H_2O_2) containing only the carbon source (citrate) and nitrogen source (glycine) for 8 hours respectively[1]. (D.) In-gel activity of isocitrate lyase from soluble CFE obtained from control and 500 µM H_2O_2 culture at the same growth phase. Gels are representative of at least 3 independent trials. *represents statistical significance in comparison to control; n=3, $p<0.05$, mean ± S.D.

[1] These values were compared to the formate levels in the control experiments where the cells were omitted respectively.

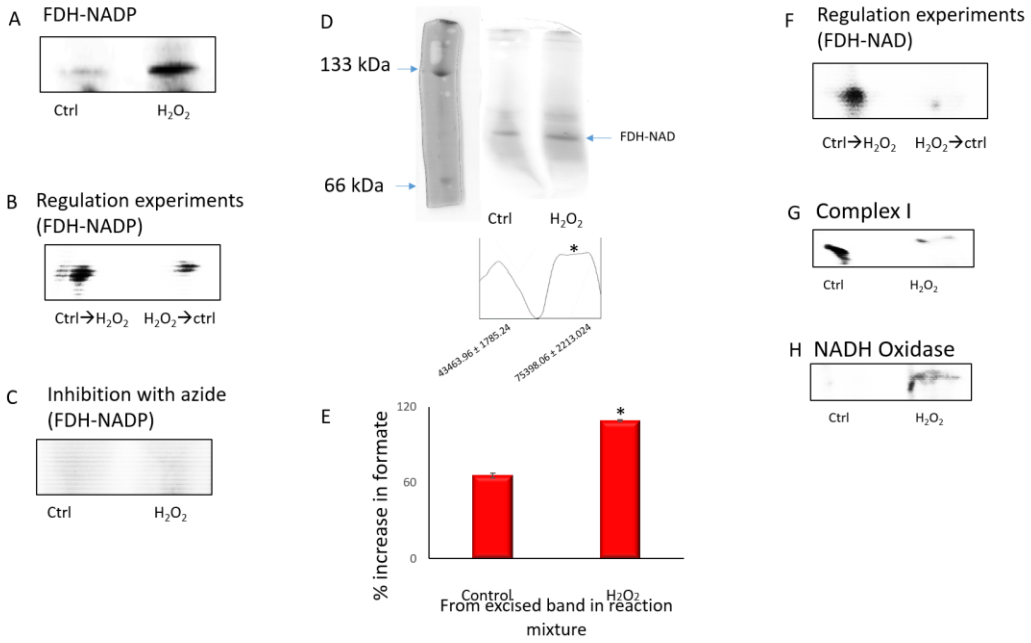

Figure 2.2: NADH homeostasis under oxidative stress. (A.) In-gel activity of FDH-NADP. (B.) In-gel activity of FDH-NADP following regulation experiments. (C.) In-gel activity of FDH-NADP using azide inhibitor to ensure specificity of enzyme. (D.) In-gel activity of NAD-dependent formate dehydrogenase. (E.) Percent increase in formate production from incubation of excised activity bands of FDH-NAD following BN-PAGE, for 30 minutes in reaction mixture containing 0.5 mM NADH and 5 mM bicarbonate[2]. (F.) In-gel activity of FDH-NAD following regulation experiments. (G.) In-gel activity of complex I. (H.) In-gel activity of NADH oxidase between control and stress cells. Gels are representative of at least 3 independent trials. *represents statistical significance in comparison to control; n=3, $p<0.05$, mean ± S.D.

[2] These are compared to the formate values in the reaction mixture in the control and stressed bands at time 0.

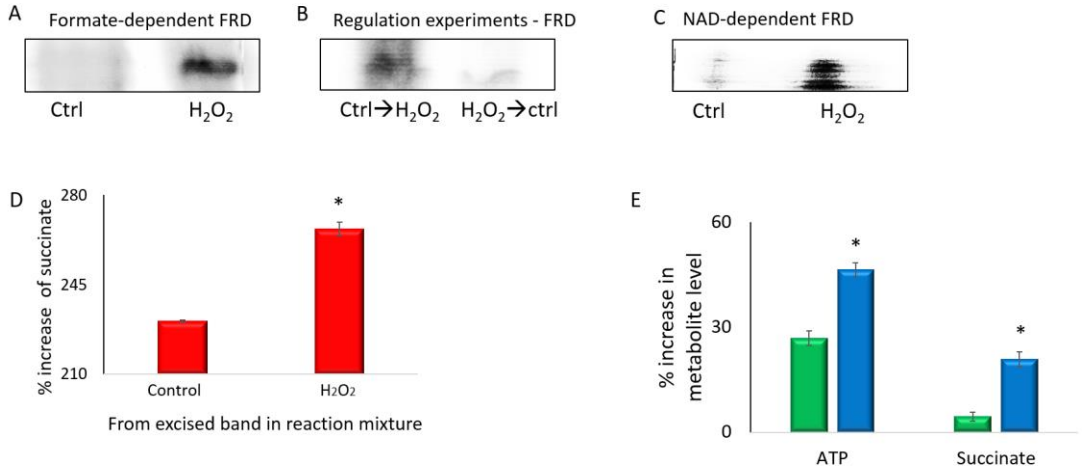

Figure 2.3: Formate-dependent succinate production. (A.) In-gel activity of formate-dependent fumarate reductase (FRD). (B.) In-gel activity of formate-dependent fumarate reductase following regulation experiments. (C.) In-gel activity of NAD-dependent fumarate reductase. (D.) Percent increase in succinate production from excised bands following BN-PAGE incubated for 30 minutes in reaction mixture containing 5 mM fumarate and 0.5 mM formate[3]. (E.) Percent increase in metabolites (ATP and succinate) from an HPLC reaction containing *Pseudomonas fluorescens* membrane fraction incubated in reaction buffer with 2 mM fumarate, 0.5 mM formate and 0.5 mM ADP for 30 minutes (control, 500µM H_2O_2). Gels are representative of at least 3 independent trials. *represents statistical significance in comparison to control; n=3, $p<0.05$, mean ± S.D.

[3] These are relative to succinate levels in the control and stress reaction bands at time 0 respectively.

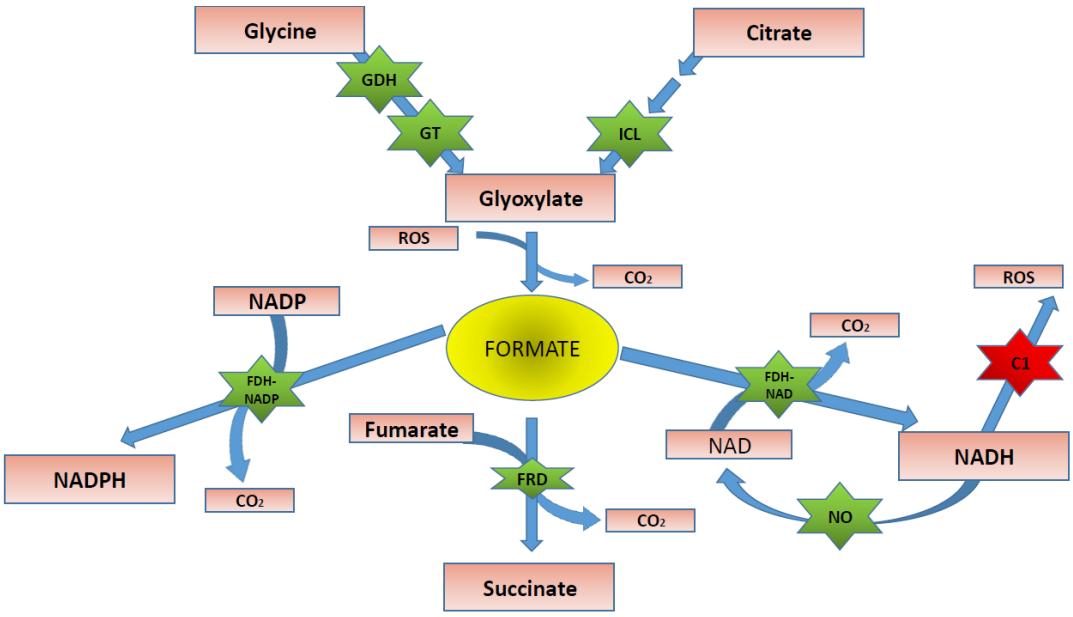

Figure 2.4: Schematic demonstrating the metabolic shift involving the role of formate in *P. fluorescens* in combatting oxidative stress (GDH: Glycine dehydrogenase; GT: Glycine transaminase; ICL: isocitrate lyase; FDH-NADP: NADP-dependent formate dehydrogenase; FRD: Fumarate reductase-formate dependent; FDH-NAD: NAD-dependent formate dehydrogenase; NO: NADH oxidase; C1: Complex I). ★=increase in enzyme activity ★= decrease in enzyme activity.

Table 1. Monitoring the activity of the select enzymes using spectrophotometric assays.

	Control	500µmol H_2O_2
ME	2.42 ± .59	4.29 ± 1.47
ICDH-NADP	1.38 ± .88	2.63 ± .29
ICDH-NAD	1.13 ± .27	.57 ± .09 *
MDH	1.78 ±.08	1.33 ± .13 *

µmol NAD(P)H produced min-1 mg protein-1 as monitored at 340nm (n=3 ± standard deviation). *represents statistical significance in comparison to control; n=3, p<0.05, mean ± S.D.

ME= malic enzyme, ICDH-NAD= NAD dependent isocitrate dehydrogenase, ICDH-NADP= NADP dependent isocitrate dehydrogenase, MDH= malate dehydrogenase).

CHAPTER 3: Conclusion, Future Research and General bibliography

3.1 Conclusion

The results from this project unveil that *P. fluorescens* grown in citrate media with glycine as the sole nitrogen source was able to thrive in spite of the negative impact of oxidative stress. In addition to modifying its metabolism to promote the formation of glyoxylate which results in increased formate production, the microbe is then able to utilize the formate in a multi-pronged antioxidative defense mechanism that promotes NADPH production, and ATP formation. This monocarboxylic acid is also invoked as a source for reducing power that is involved in a variety of enzymatic reactions thus, obviating the need for NADPH and NADH. The latter is a pro-oxidant and its diminished synthesis would aid in impeding intracellular ROS formation.

FDH-NADP, which was markedly increased in the stressed cells might help with the increase of NADPH which is a critical component of the antioxidative defense mechanisms in these organisms (Beriault et al., 2005, Beriault et al., 2007). Additionally, enzymes like ME and ICDH NADP also displayed enhanced activity in the stressed cells, an observation that is indicative of the importance in NADPH in antioxidative defense strategies.

NADH producing enzymes such as ICDH-NAD and MDH were found to be diminished in the stressed cells consistent with previous studies (Alhasawi et al., 2015). However, the activity of FDH-NAD was in fact found to be augmented in the stressed cells. It is postulated that the added NADH would prove beneficial in the reduction of fumarate to succinate via FRD which was also found to be up-regulated. However, it is evident that the organism has opted for enhanced

production of NADPH and diminished formation of NADH. This metabolic reprogramming not only helps combat oxidative stress via NADPH but also limits the intracellular generation of ROS through the activity of the pro-oxidant, NADH. The diminished NADH synthesis appears to be compensated by the utilization of formate as a reducing co-factor. Enzyme like fumarate reductase readily converts fumarate into succinate in the presence of formate. Thus, formate produced seemingly as a byproduct of ROS detoxification with the non-enzymatic decarboxylation of glyoxylate may in fact contribute to the antioxidative defense mechanism via the generation of NADPH but also acts as a potent reductive force generating key metabolites to enhance the viability of the bacteria. This study revealed the significance of metabolic reprogramming in anti-oxidative defense and demonstrated the critical role keto-acids play in diminishing oxygen tension.

3.2 Future work

Future work emanating from these findings can be aimed at examining formate homeostasis in cellular systems exposed to oxidative stress. This study may be accomplished via the use of labelled citrate or labelled glycine in separate experiment to delineate which of these carbon sources contributes to the maximal formation of formate. Additionally, the participation of formate as reducing factor in various enzymes like ribonucleotide reductase can also explored. Arguably alternate pathways leading to formate synthesis under stressed condition can also be evaluated. Formate is being used to make energy as well as a substitute of NADH/NADPH. Other

enzymes such as ribonucleotide reductases which could potentially utilize formate in lieu of NADH/NADPH to drive metabolism can be studied.

Furthermore preliminary data show that formate may also play a role in the detoxification of nitrosative stress. Indeed, NO_2/NO_3 reductases were found to have elevated activity in cells stressed with sodium nitroprusside (SNP), a generator of reactive oxy-nitrosative species (RONS). Future studies can also be devoted to the mechanisms by which this microbe combats reactive nitrogen species (RNS) and also to delineate whether or not formate is being used in the energy-making machinery and contributing to the NADH/NADPH balance. This will help establish a universal role of formate in neutralizing oxidative stress, especially in the light of the recent findings that correlate the levels of this monocarboxylic acid in blood to hypertension in humans (Holmes et al., 2008).

3.3 General Bibliography

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