Metabolic networks dedicated to the generation of the antioxidant pyruvate and ATP enable the survival of *Pseudomonas fluorescens* in an oxidative environment

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Abstract

As all aerobic organisms are exposed to oxidative stress, they have evolved intricate mechanisms to control reactive oxygen species (ROS). Antioxidants such as glutathione along with ROS detoxifying enzymes like catalase and superoxide dismutase (SOD) have been widely reported in literature. Recently our laboratory has uncovered the role of the ketoacid, α-ketoglutarate in ROS scavenging. In this present work, the importance of metabolism in generating pyruvate, another potent ketoacid antioxidant, and in enabling the production of ATP despite an ineffective oxidative phosphorylation machinery is shown. The soil microbe, Pseudomonas fluorescens grown on glucose in the presence of 100μM hydrogen peroxide (H₂O₂) presented significant oxidized cellular components in comparison to the control cultures. Levels of pyruvate and acetate were found to be higher in the soluble cell-free fraction and the spent media of the H₂O₂-stressed cells. The latter is generated when pyruvate is involved in the scavenging of ROS. Pyruvate accumulation was promoted by a decrease in pyruvate dehydrogenase (PDH) activity, a phenomenon that limited the supply of acetyl-CoA for the tricarboxylic acid (TCA) cycle. Acetate kinase (AcK) activity increased concomitantly with the concentration of acetate in the cell-free extract of stressed P.fluorescens. As oxidative phosphorylation was hindered by H₂O₂, substrate level phosphorylation (SLP) through phosphoenolpyruvate synthase (PEPS) was shown to be a source of ATP for the cells grown in the
oxidative environment. The increased activities of adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) were involved in maintaining this ATP-generating system. The metabolism of glucose via glucose dehydrogenase (GDH)-NADP dependent and the pentose phosphate pathway (PPP) contributed to the generation of NADPH and pyruvate in the stressed cells. The participation of NADH oxidase (NADHOX) and NAD kinase (NADK), allowed for the metabolic shift towards the increased production of NADPH and the decreased synthesis of NADH. Metabolism is the foundation of any cellular adaptation; hence its modulation is pivotal for any survival strategy. Here, the metabolic adaptations aimed at generating pyruvate and ATP provide \textit{P. fluorescens} with two important tools to combat a \textit{H}_2\textit{O}_2^- enriched environment.
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List of abbreviations

6PG – 6-phosphogluconate
6PGDH – 6-phosphogluconate dehydrogenase
AA – ascorbic acid
ACC – acetyl CoA carboxylase
AcK – acetate kinase
AD – Alzheimer’s disease
ADP – adenosine 5’ diphosphate
AGODH – acetylating-glyoxylate dehydrogenase
AK – adenylate kinase
α-KG – alpha-ketoglutarate
AMP – adenosine 5’ monophosphate
APS – ammonium persulfate
ATP – adenosine 5’ triphosphate
BN PAGE – blue native polyacrylamide gel electrophoresis
BSA – bovine serum albumin
CAT – catalase
CFE – cell-free extract
CK – creatine kinase
CoA – coenzyme A
CVDs – cardiovascular diseases
Cyt C – cytochrome c
DCIP – dichloroindophenol
DNPH – dinitrophenylhydrazine
DTT – dithiothreitol
ETC – electron transport chain
FAPy – formimidopyrimidines
FUM – fumarase
F6P – fructose-6-phosphate
G3P – glyceraldehyde-3-phosphate
G6P – glucose-6-phosphate
G6PDH – glucose-6-phosphate dehydrogenase
GDH – glucose dehydrogenase
GDP – guanosine 5’ diphosphate
GPI – glucose-6-phosphate isomerase
GR – glutathione reductase
GS(H) – glutathione
GST – glutathione transferase
GTP – guanosine 5’ triphosphate
HK – hexokinase
HMS – hexose monophosphate shunt
HNE – 4-hydroxynonenal
HPLC – high performance liquid chromatography
ICDH – isocitrate dehydrogenase
ICL – isocitrate lyase
INT – iodonitrotetrazolium
KGDH – α-ketoglutarate dehydrogenase
LDH – lactate dehydrogenase
LMWA – low molecular weight antioxidants
MAPK – mitogen-activated protein kinase
mCFE – membrane cell free extract
MDA – malonaldehyde
MDH – malate dehydrogenase
ME – malic enzyme
MS – multiple sclerosis
NAD – nicotinamide adenine dinucleotide (oxidized)
NADP – nicotinamide adenine dinucleotide phosphate (oxidized)
NADH – nicotinamide adenine dinucleotide (reduced)
NADPH – nicotinamide adenine dinucleotide phosphate (reduced)
NADHOX – NADH oxidase
NADK – NAD kinase
NDPK – nucleotide diphosphokinase
OCT – oxalate CoA transferase
PC – pyruvate carboxylase
PD – Parkinson’s disease
PK – pyruvate kinase
PDH – pyruvate dehydrogenase
PEP – phosphoenolpyruvate
PEPS – PEP synthase
PHD – prolyl hydroxylases
PMS – phenazine methosulfate
PPP – pentose phosphate pathway
PTP – protein tyrosine phosphatase
PUFAs – polyunsaturated fatty acids
RNS – reactive nitrogen species
ROS – reactive oxygen species
sCFE – soluble cell free extract
SCS – Succinyl-CoA synthetase
SD – standard deviation
SDH – succinate dehydrogenase
SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLP – substrate level phosphorylation
SOD – superoxide dismutase

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SOR – superoxide reductase
TBA – thiobarbituic acid
TBARS – thiobarbituic acid reactive species
TCA – tricarboxylic acid
Tris – tris(hydroxymethyl)aminomethane
1. Introduction

It is estimated that approximately 2 billion years ago, a significant increase in oxygen levels in the earth’s atmosphere occurred. The majority of this oxygen originated from photosynthetic life, which began long before the presence of molecular oxygen in the air. Most of the initial oxygen produced by cyanobacteria was consumed by the oxidation of surface materials, a process known as chemical weathering (Canfield, 2005). It was not till about 400 million years ago, when the sources of free metal reactants were exhausted, that the generation of a stable oxygenated atmosphere was enabled (Kump, 2008). Consequently, the advent of oxygen in the atmosphere instigated the evolution of living organisms dependent on molecular oxygen in their combustion of reduced substrates, a process termed respiration. Oxidative phosphorylation evolved and propelled aerobes to thrive all across the planet (Canfield, 2005; Dismukes et al. 2001; Kasting et al. 2002; Kump, 2008). Like a veritable “double edge sword”, using oxygen as a terminal electron acceptor also generates a highly oxidative and potentially toxic cellular environment.

1.1. The oxidizing power of oxygen

There are a wide variety of oxygen species found in biological systems
and some show significant reactivity with biomolecules, which can lead to the disruption of cellular functions. These are known as Reactive Oxygen Species (ROS) (Apel 2004). These potentially harmful moieties are generated by energy transfer or when ground state triplet oxygen undergoes sequential univalent reduction (figure 1).

**Figure 1.** Some biologically relevant reactive oxygen species derived from the ground-state triplet oxygen molecule. Dioxygen can accept one electron to form the superoxide radical anion. A second reduction in the presence of protons will lead to hydrogen peroxide formation. Ultimately, reduction of oxygen will lead to water formation (Modified from Apel, 2004).

Due to its electronic configuration, dioxygen is an avid electron acceptor, giving it characteristic oxidant properties. However, even though oxygen possesses significant oxidizing power its reactivity is surprisingly low, which explains why the formation of water does not occur spontaneously when triplet oxygen and dihydrogen are added together. Since oxygen has an even number of electrons, it comprises two non-paired electrons in its molecular orbitals (figure 2). These two electrons have the same quantum spin.
numbers, indicative of parallel spins, which are demonstrated as parallel arrows in the two highest energy molecular orbitals in figure 2. In order to oxidize molecules by accepting an electron pair, dioxygen would need to react with a molecule which also has parallel spinning electrons in order to pair up with those of this oxidant. This rule is known as the "spin-verbot". The feature of parallel spins is unusual and therefore oxygen's reactivity is said to be "spin-forbidden" with many biomolecules. For these reasons, oxygen is limited to single-electron transfer reactions which leads to the superoxide anion radical ($O_2^-$), hydrogen peroxide (H$_2$O$_2$) and the highly reactive hydroxyl radicals (-OH), with respect to the order of reduction (Ziegelhofer, 2009).

![Molecular orbitals diagram](image)

**Figure 2.** Molecular orbital diagram of ground state triplet oxygen (Ziegelhofer, 2009).
1.2. Sources of ROS

Since molecular oxygen is relatively abundant (20.9%) in the Earth’s atmosphere, there is a vast source of ROS being produced through continuously occurring reactions. For example, many cellular processes in aerobic cells can cause an accumulation of reactive by-products such as $\text{O}_2^{-}$, $\text{H}_2\text{O}_2$ and ·OH. The majority of biologically-derived $\text{O}_2^{-}$ is generated by inefficient electron transfer between membrane-associated respiratory chain enzymes, also known as the electron transport chain (ETC) (figure 3) (Liu, 2002; Seifert, 2010). The ETC is involved in the process of oxidative phosphorylation utilized by aerobic organisms to generate energy in the form of adenosine triphosphate (ATP) (Lushchak, 2001). In eukaryotic cells, the ETC is generally located in the mitochondria where ATP is produced from products of the tricarboxylic acid (TCA) cycle, fatty acid oxidation and amino acid oxidation. In the mitochondrial membrane, electrons are transferred from succinate or NADH through the ETC to oxygen, which is subsequently reduced to water (Seifert, 2010). The chain, as the name suggests, consists of a group of enzymatic electron acceptors and donors. Each electron donor passes electrons to a more electronegative acceptor, which will in turn donate the electrons to the following acceptor. This process continues down the series of enzymes until electrons are passed to the most electronegative and terminal electron acceptor, oxygen. Through the passage of electrons between donor and acceptor, there is a release of energy, which is used to
form a proton gradient across the mitochondrial membrane. This gradient produces an energy potential that is harnessed by the enzyme ATP synthase to produce ATP.

![Diagram of the electron transport chain](image)

**Figure 3.** The electron transport chain of oxidative phosphorylation is a source of ROS (Mailloux, 2011).

In prokaryotes, the ETC is more diverse, with a variety of different electron donors and acceptors being used. Depending on the electron donors, bacteria use dehydrogenases (such as succinate dehydrogenase or NADH dehydrogenase), quinones (such as ubiquinone) and cytochromes to carry the electrons through the ETC (Anraku, 1988). What is universal and essential for oxidative phosphorylation is the pumping of protons across a cell membrane in order to create the necessary electrochemical gradient for ATP production.
In eukaryote and prokaryotes alike, the transfer of electrons through the chain is not always efficient. There are a small percentage of electrons that do not follow the proper path but rather leak directly towards oxygen, resulting in the formation of the free-radical superoxide. Under physiological conditions the superoxide radical is rapidly transformed into \( \text{H}_2\text{O}_2 \) by a dismutation reaction. This reaction, although spontaneous, is usually catalyzed by the enzyme superoxide dismutase (SOD). In *Escherichia coli*, experimental data have shown that up to 87% of the total \( \text{H}_2\text{O}_2 \) produced in the bacterium is attributed to the ETC. The steps responsible for the single electron reduction of oxygen in this gram-negative bacteria are at the NADH dehydrogenase and ubiquinone points of the respiratory chain. Further ROS production occurs when \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) both participate in the formation of the hydroxyl radical via the Haber-Weiss and Fenton reactions (figure 4) (Gonzalez-Flecha, 2006).

**Haber-Weiss reaction:**

\[
\text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+}
\]

**Fenton reaction:**

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^+ + \text{Fe}^{3+}
\]

**Figure 4.** Superoxide and hydrogen peroxide are both involved in the generation of the hydroxyl radical through the Haber-Weiss and Fenton reactions. Iron in these reactions can be replaced with copper. (Gonzalez-Flecha, 2006)

Although the respiratory chain is a major contributor to ROS production
in a cell, there are many other intracellular sources that can elevate the levels of these toxic moieties. Many enzymes like xanthine oxidase, glucose oxidase and NADPH oxidase all produce H₂O₂ as a byproduct of their reactions (Förstermann, 2009). Aside from enzymes, there are chemical reagents known to be redox-cycling agents such as menadione and paraquat. When internalized, these compounds generate intracellular O₂⁻ which increases the oxidative burden faced by the cell. Menadione (2-methyl-1,4-naphthoquinone) is a quinone often used in the study of oxidative stress and is involved in plant biochemistry as a chemical defense agent. Quinones, which compose a prominent fraction of the structure of pigments, antibiotics, vitamin K and coenzymes, play many biological functions, notably in the one-electron transfer agents in aerobic metabolism. In living systems, menadione can undergo one electron reduction by enzymes such as microsomal cytochrome p-450 reductase, consequently transforming it into a semiquinone radical. Under aerobic conditions, this semiquinone radical participates in redox-cycling to generate ROS like O₂⁻ (figure 5) (Castro, 2008; Criddle, 2006).
Figure 5. Metabolism of menadione by one-electron reducing enzymes generates an unstable semiquinone radical, with further reduction to the stable hydroquinone. Back-oxidation generates ROS (O$_2^-$) when molecular oxygen is present (Modified from Criddle, 2006).

Furthermore, quinones are potent electrophiles, capable of reacting with the thiol groups of proteins and glutathione (GSH). Menadione-induced cytotoxicity and oxidative stress have been associated with the generation of GS-conjugates catalyzed by glutathione S transferase isoforms (GST) leading to the depletion of GSH (Castro, 2008).

Along with chemical reagents, ionizing and near-UV radiation can also
cause the formation of free-radicals within a cell. The major contributor of ROS stress induced by ionization is the hydroxyl radical which can be generated by the homolytic fission of water whereas UV light can produce singlet oxygen and \( \text{H}_2\text{O}_2 \) in reactions involving chromophores (Leach, 2001; Stankovic, 2008). According to a study done by Narayanan et al. (1997), exposing cells to either \( \alpha \)-particles or \( \gamma \)-radiation significantly enhanced cellular ROS/RNS (reactive nitrogen species) levels above that formed by normal cell metabolism. Experiments with dihydroethidium, a fluorescent dye sensitive to superoxide anion, confirmed \( \alpha \)-particle radiation-induced ROS generation (Narayanan, 1997).

1.3. Biological targets of ROS

As the name “reactive” oxygen species suggests, these oxygen derivatives tend to exert high reactivity with biomolecules such as proteins, lipids and nucleic acids. This accounts for their involvement in cellular toxicity and diseases.

1.3.1. Oxidation of lipids by ROS

Lipids are considered one of the major targets for ROS. Noteworthy are the polyunsaturated fatty acids in cellular membrane that easily react with free radicals, thus initiating the process known as lipid peroxidation. Lipid
peroxidation, a radical chain reaction, can hinder the quality of the membrane. It hinders membrane fluidity, ultimately leading to altered membrane properties and the disruption of membrane-bound proteins. In addition, peroxidation acts as an amplifier of radical formation. Once initiated by ROS, lipid peroxidation produces more radicals and leads to the degradation of polyunsaturated fatty acids (PUFAs) (figure 6) (Grimsrud, 2008; Halliwell, 1993). Some of these products, such as aldehydes, are highly reactive towards molecules such as proteins and other lipids. Aldehydes differ from reactive free radicals as they are significantly long lived allowing them to diffuse from the site of their origin to reach distant macromolecules far from the initial free-radical event. Aldehydes act as "second toxic messengers of the complex chain reactions initiated" (Ros, 2000).

Among the many different aldehydes which can form during lipid peroxidation, the most intensively studied are malonaldehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (HNE) (Briganti, 2003; Grimsrud, 2008). The primary instigator of lipid peroxidation is the hydroxyl radical, which is generally produced in-vivo by the decomposition of peroxynitrite (ONOO-) and by the reactions of transition-metal ions with $\text{H}_2\text{O}_2$ (Halliwell, 1993; Sayre, 2008). $\cdot\text{OH}$ is without a doubt more reactive than $\text{H}_2\text{O}_2$ and $\text{O}_2\cdot^-$ and therefore responsible for the majority of the cellular damage associated with oxidative stress. The hydroxyl radical initiates lipid...
Figure 6. Lipid peroxidation reactions.

peroxidation by abstracting a hydrogen atom from fatty acids or fatty acyl side chains. The greater the number of double bonds in a fatty acid side chain, the easier is the removal of a hydrogen atom. This is why PUFAs are particularly susceptible to peroxidation. The removal of the hydrogen leaves behind an unpaired electron on the carbon atom. The resulting carbon-centered lipid radical can react in various manners, of which molecular rearrangement is most likely. This rearrangement can be followed by reaction with H₂O₂ to give
a peroxyl radical. Peroxyl radicals either combine with other peroxyl radicals, or they can attack membrane proteins (Grimsrud, 2008). It is also possible that a peroxyl radical propagates the lipid peroxidation chain reaction by removing a hydrogen from an adjacent fatty acid. Consequently, a single initiation event can result in conversion of hundreds of fatty acid side chains into lipid hydroperoxides (Halliwell, 1993).

1.3.2. Nucleic acid damage caused by oxidative stress

Cellular components that are in proximity of ROS generation sites are more likely subjected to their reactivity. This is noticeable within the cell in areas where free transition metal ions are found. A clear demonstration of this situation is damage caused to DNA by ROS. This specific type of assault carried out by ROS is very important since DNA modifications can result in point mutations and in the case of higher organisms, malignant cell transformations (Darzynkiewicz, 2006). An example of the relationship between the localization of a cell component and the odds of reacting with ROS is seen in fibroblast exposed to H$_2$O$_2$. The induced oxidative stress first modifies mitochondrial but not nuclear DNA. Calculations suggest that mitochondrial DNA is damaged 104 times more frequently than nuclear DNA (Lushchak, 2001). This phenomenon is attributed to the fact that mitochondrial components are subjected to oxidative stress more intensively than nuclear elements seeing as endogenous ROS are generated mainly by
the ETC (Lushchak, 2001; Ritcher, 1988).

In prokaryotes, there is an absence of strict spatial organization for oxidative processes such as oxidative phosphorylation, leading to the wide spectrum of viable targets of radical oxygen derivatives. DNA is always an attractive target for the hydroxyl radical and therefore damage to nucleic acids occurs via the same mechanisms as in eukaryotes. As Fe(II) is present at the site of the phosphodiester groups of nucleic acids, \( \text{H}_2\text{O}_2 \) and this free metal ion can easily react to form the hydroxyl radical which can introduce lesions to the DNA chain. Oxidative damage to nucleic acids include: adducts of base and sugar groups, single- and double-strand breaks in the backbone, and cross-links to other molecules. The spectrum of adducts in oxidized DNA in vitro and in vivo includes more than 20 known products, including the damage to all four bases and thymine-tyrosine cross-links (Ros, 2000).

1.3.3. The effect of oxidizing agents on protein structure, function and integrity.

When proteins are exposed to reactive oxygen species, modifications to the side chain of amino acids can take place, leading to structure modification in the protein. These protein modifications result in functional changes that alter cellular metabolism. Changes in the reducing environment of the cytosol can have significant effects on protein since the redox state of the cytoplasm
will affect protein folding and activity (Ghezzi, 2005). Thiols of cysteines in proteins are the major targets of H$_2$O$_2$ (Leichert, 2008). The reaction of cysteinyil thiolates with H$_2$O$_2$ can lead to the formation of different transformations, such as sulfenic acid (−SOH), sulfinic acid (−SO$_2$H), and sulfonic acid (−SO$_3$H), as well as disulfide bond formation (−S−S−) and glutathione conjugation (−S−GSH) (Figure 7) (Kiley, 2004).

![Diagram of thiol modification by hydrogen peroxide](image)

**Figure 7.** Thiol modification by hydrogen peroxide first leads to the formation of a sulfenic acid (Kiley, 2004).

Thiol modifications are linked to alterations in the structure and function of proteins. A common reoccurrence has been observed in the chemical reaction of H$_2$O$_2$ with thiolates, this being the initial formation of sulfenic acid. In the case of proteins that have a nearby cysteinyil residue, a disulfide bond forms between the two sulfur atoms (figure 7). The sulfenated cysteinyil residue can also react with a cysteinyil residue on another protein or with
glutathione, giving rise to a mixed disulfide (figure 7). If no cysteiny1 residue is nearby, the sulfenated cysteine can be further oxidized to sulfinic or sulfonic acid (figure 7), or it can remain in the sulfenic acid state (figure 7). As most forms of thiol oxidation are reversible it has been suggested that thiol modifications contribute to the signal transduction processes in a manner similar to protein phosphorylation/dephosphorylation (Kiley, 2004; Poole, 2004).

In the presence of oxidizing agents, irreversible oxidation of amino acid residues in a protein has been observed. The hydroxyl radicals can react with an α-hydrogen atom of an amino acid residue to form a carbon-centered radical. In the presence of oxygen peptide bond cleavage occurs via the formation of an alkoxy radical. If oxygen is absent, the carbon-centered radical may react with another one to form protein-protein cross-linked derivatives. According to Ros, "the main consequences of protein modifications observed under oxidative exposure are: loss of catalytic activity, amino acid modifications, carbonyl group formation, increase in acidity, decrease in thermal stability, change in viscosity, change in fluorescence, fragmentation, formation of protein-protein cross-links, formation of S–S bridges, and increased susceptibility to proteolysis" (Table 1) (Ros, 2000).

ROS-mediated protein alterations that lead to the loss of catalytic
activity of a protein can have serious effect on cellular metabolism. It has been observed that a number of enzymes with iron-sulfur clusters active sites are highly sensitive to inactivation by O$_2^-$ (D’Autréaux, 2007; Djaman, 2004). Iron-sulfur clusters [Fe-S], as the name suggests, are clusters of non-heme

Table 1. Amino acid residues of proteins that are oxidized and products formed (Ros, 2000).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Oxidation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>Glutamic semialdehyde</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Disulfides, cysteic acid</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Oxalic acid, pyruvic acid</td>
</tr>
<tr>
<td>Histidine</td>
<td>2-Oxohistidine, asparagine, aspartic acid</td>
</tr>
<tr>
<td>Lysine</td>
<td>2-Aminoadipic semialdehyde</td>
</tr>
<tr>
<td>Methionine</td>
<td>Methionine sulfoxide, methionine sulfone</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine</td>
</tr>
<tr>
<td>Proline</td>
<td>2-Pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid, glutamic semialdehyde</td>
</tr>
<tr>
<td>Threonine</td>
<td>2-Amino-3-ketobutyric acid</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2-, 4-, 5-, 6-, and 7-hydroxytryptphan, nitrotyptphan, kynurenine, 3-hydroxykynurenine, formylkynurenine</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3,4-Dihydroxyphenylalanine, Tyr-Tyr cross-linkages, Tyr-O-Tyr, cross-linked nitrotyrosine</td>
</tr>
</tbody>
</table>

iron and inorganic sulfide and are considered as one of the most ubiquitous and functionally versatile prosthetic groups in nature. [Fe-S] clusters have the ability to delocalize electron density over both Fe and S atoms rendering them ideal for their primary role in mediating biological electron transport. For this reason, [Fe-S] clusters are major components in the photosynthetic and respiratory ETC, they are the foundation of pathways of electron flow in numerous membrane-bound and soluble redox enzymes and constitute the redox-active centers in ferredoxins, one of the largest classes of mobile
electron carriers in biology. The vast majority of electron transfer [Fe-S] clusters are one-electron carriers. In a wide range of redox and non-redox enzymes, [Fe-S] clusters also participate, in whole or in part, in substrate-binding. An example of a catalytic, [Fe-S] containing enzyme is aconitase. The [Fe-S] cluster of aconitase is characterized by a non-cysteinyl ligation at a particular Fe site of a [4Fe-4S] cluster which allows it to facilitate substrate binding and activation for dehydration/hydration reactions (Johnson, 2005). What is particularly interesting about aconitase is its extreme vulnerability to oxidation by small oxidants that can reach the active site. Such oxidants include the O$_2^-$, H$_2$O$_2$ and peroxynitrite (ONOO$^-$). The mechanism of aconitase inhibition by O$_2^-$ involves the release of free iron from the enzyme (figure 8), which indirectly adds to the oxidative burden of a cell (as mentioned previously with the Fenton reaction) and directly affects the cluster's ability to interact the leaving hydroxyl group of the dehydration substrate, rendering the enzyme inactive (D'Autréaux, 2007). The release of free iron has been proposed to account for the genotoxicity of superoxide radicals (Ros, 2000).
\[ \text{Ox} + [4\text{Fe}-4\text{S}]^{2+} \rightarrow \text{Red} + [4\text{Fe}-4\text{S}]^{3+} \]

**Reaction 1**

\[ [4\text{Fe}-4\text{S}]^{3+} \rightarrow [3\text{Fe}-4\text{S}]^{1+} + \text{Fe}^{2+} \]

**Reaction 2**

**Figure 8.** Iron-sulfur clusters react with small oxidants such as superoxide (reaction 1). Oxidation converts the clusters to an unstable form that degrades rapidly (reaction 2) liberating a free iron ion (Djaman, 2004).

It is well established now that the mild oxidation of proteins yields better substrates for proteolytic digestion. Since modified proteins often experience a significant loss of function, they have to be replaced by the cellular protein synthesis machinery. To avoid excessive accumulation of damaged proteins, there are specific proteolytic systems in place to degrade the oxidized substrates. In bacterial systems, like in aerobically-grown *E.coli*, specific proteinases selectively degrade oxidized proteins in an ATP-independent manner. In eukaryotic cells, the multi-catalytic proteinase complex called the proteasome is responsible for the degradation of many intracellular proteins in a non-lysosomal mediated pathway. It has been suggested that protein oxidation could prime the substrate for ubiquitination, which in turn would signal for proteasomal degradation. The degree of protein oxidation plays an important role in signaling for degradation and it appears that past a certain threshold the oxidation level of a protein can lead to detrimental effects on the proteasome's task. A significant body of literature indicates that severe
oxidation stabilizes proteins due to aggregation, cross-linking, and/or decreased solubility, thus increasing their half-lives. The inability to degrade extensively oxidized proteins may contribute to certain disease states and has been postulated as one of the causes of aging (Grune, 2003; Petropoulous, 2006; Ros, 2000).

1.4. ROS, disease and aging

The high reactivity of oxygen and of its reduced derivatives imposes a significant burden on all living organisms. The fact that no biomolecule is safe from the attack of ROS makes them a constant threat in all cells. As properly functioning cells require selectively permeable lipid membranes, depend on enzymes for the metabolism of nutrients, and rely greatly on the integrity of DNA for the functionality of gene-coded proteins, it is easy to see the relationship between ROS and diseases. The consequence of proteins, lipids and nucleic acids reacting with ROS can be disastrous if not counteracted. In microbes, high levels of ROS can be bactericidal and in higher organisms the inefficient regulation of active oxygen has been associated with diseases affecting all organs. Studies on neurodegenerative diseases such as Alzheimer’s disease (AD), Multiple sclerosis (MS) and Parkinson disease (PD), have all pinpointed ROS as a key factor in the mechanisms of the development of these conditions. The role of ROS toxicity in the development of these diseases include the release of free transition elements like iron (e.g.
in PD), the initiation of protein aggregation (i.e. in AD), and ultimately the activation of apoptosis leading to cell death (Dar, 2009; Lin, 2006). Along with neurological dysfunctions, cardiovascular diseases (CVDs) represent another set of conditions where severe endothelial injury is linked to oxidative species. Common CVDs that have pathological hallmarks caused by ROS include hypertension, atherosclerosis, ischemic heart disease, cardiomyopathies and congestive heart failure (Fürstermann, 2009). Other pathologies linked to oxidative stress are inflammation, cystic fibrosis, diabetes and cancers (Benz, 2008).

Although diseases represent an important aspect of the risks associated with living in an oxidative environment, a more universally natural phenomenon has been linked to ROS. In the early 1900’s it was observed that animals with higher metabolic rates often have shorter life spans. These observations provided the foundation for the “rate of living hypothesis” which postulates that the metabolic rate of a species ultimately determines its life expectancy. In the 1950’s, Denham Harman theorized the “free-radical theory of aging” explaining that endogenous ROS generated by the cell would cause cumulative damage. Given that the mitochondria is the site of the ETC, where energy is harnessed from the breakdown of carbon sources, and correspondingly consume the bulk of intracellular oxygen, the free-radical theory of aging is now often thought of when discussing the rate-of-living hypothesis. The higher the metabolic rate of an organism, the greater the
production of ROS and hence the shorter the life span. This observation is not true for all species. In some, the correlation between metabolic rate and life span is not maintained. Noteworthy are birds and primates, which tend to live longer than would be predicted by their metabolic rates. Careful analysis of oxidant production demonstrated that at a given metabolic rate, mitochondria from these species tend to produce fewer ROS. This indicates that ROS production rather than metabolic rate provides the strongest correlation with overall longevity (Benz, 2008; Finkel, 2000).

1.5. ROS: essential and destructive

Although dangerous at high doses, somewhere along the evolutionary road, organisms achieved a high degree of control over ROS toxicity thus allowing them to integrate these free-radicals in many cellular processes. The use of ROS in normal cellular function is a field of study gaining in importance and is one that shows the resilience and the adaptive behavior of life in the face of such a toxic, oxygenated environment. All phyla of life must tolerate ROS, whether it be plants, animals, fungi or bacteria, they all use the reactivity of these activated moieties to their advantage (Harir, 2009).

1.5.1. The role of ROS in signaling

It has been observed in eukaryotes that ROS generation in cellular
compartments such as the mitochondria or chloroplasts results in changes to the nuclear transcriptome, indicating that this information must be transmitted from these organelles to the nucleus, but the identity of the transmitting signal remains unknown. This concept is named REDOX signaling. Three principal modes of action indicate how ROS may affect gene expression (figure 9).

ROS sensors may be activated to induce signaling cascades that ultimately manipulate gene expression. Alternatively, components of signaling pathways may be directly oxidized by ROS. Finally, ROS may change gene expression by targeting and modifying the activity of transcription factors. It is important to realize that different ROS will react differently with cellular components and therefore will induce different responses from the cell (Apel, 2004; Harir, 2009; Miller, 2008).

1.5.1.1. ROS Sensing by Histidine Kinases

In prokaryotes and fungi, communication between the extracellular environment and the cytoplasm is essential for proper proliferation. These organisms are equipped with two-component signaling systems that function as redox sensors. By assessing the redox state of their environment, cells can tailor their activities to adequately deal with the pressures of their surroundings. In prokaryotes, the two-component signaling systems usually consist of a histidine kinase that senses the signal and a response regulator that functions as a transcription factor. The transmembrane kinase draws its
sensory function from its capacity to autophosphorylate a histidine residue in response to an external stimulus. Following autophosphorylation, the phosphoryl group is transferred to an aspartate residue in the response regulator. The induced conformational change in the response regulator alters its DNA-binding affinity and thus promotes gene expression from certain promoters (Apel, 2004).

Although histidine kinases are part of two-component signal transduction systems in prokaryotes that act on their own, sensors found in fungi and plants are part of more elaborate pathways (Foyer, 2005). For example, the yeast Sln1 kinase transfers its phosphoryl group through the intermediate Ypd1 to its final destination in the response regulator Ssk1. Autophosphorylation of Sln1 is inhibited by stress which leads to the accumulation of the non-phosphorylated form of Ssk1 which in turn activates the Hog1 mitogen-activated protein kinase (MAPK) cascade. MAPK pathways are responsible for signaling cascades induced by a wide variety of stresses including ROS, along with their role in hormone response and cytokinesis (Gustin 1998).
Figure 9. Schematic depiction of cellular ROS sensing and signaling mechanisms. ROS sensors such as membrane-localized histidine kinases can sense extracellular and intracellular ROS. Intracellular ROS can also influence the ROS-induced mitogen-activated protein kinase (MAPK) signaling pathway through inhibition of MAPK phosphatases (PPases) or downstream transcription factors. Whereas MAP kinases regulate gene expression by altering transcription factor activity through phosphorylation of serine and threonine residues, ROS regulation occurs by oxidation of cysteine residues (Modified from Apel, 2004).
1.5.1.2. ROS Inhibition of Protein Phosphatases via thiol oxidation

Cysteine residues offer a regulatory mechanism of signal transduction due to the fact that the oxidation of cysteine side chains of proteins render the latter more susceptible to modifications by S-glutathionylation. S-glutathionylation is a reversible post-translational modification that, first of all, offers protection of protein cysteine from irreversible oxidation. Secondly, S-glutathionylation can serve as a mediator of redox signal transduction. Adding glutathione to a protein can induce conformational changes rendering the targeted molecule inactive or, alternatively, it can activate certain signaling cascades (Gallogly, 2007; Poole, 2004).

The reactive oxygen species H₂O₂ can oxidize thiol residues. This event, which leads to the modification of the thiol groups of a protein, can subsequently be sensed. The effects of protein oxidation are not limited to sensing the redox state of the environment but they also act on signaling pathways. This fact can be seen in studies that have shown that human protein tyrosine phosphatase (PTP) 1B can be modified by H₂O₂ at the active site cysteine (Van Montfort, 2003). Inactivation of PTP1B by H₂O₂ is reversible and can be brought about by incubation with glutathione. A similar regulation likely occurs in plants because PTP1, an Arabidopsis PTP that can inactivate the MAPK signaling cascade intermediate MPK6 in Arabidopsis,
can be inactivated by $\text{H}_2\text{O}_2$ (Gupta, 2003).

1.5.2. Oxidative burst in macrophages of the immune system

Phagocytic cells of the mammalian immune system such as neutrophils and macrophages are responsible for the destruction of pathogen-infected cells or engulfed microbes in a host organism. These cells contain an array of mechanisms to rid the infected body of the invading bacteria. A well-studied strategy evoked by phagocytes is the stimulated production of ROS, a process dubbed “the respiratory burst”. The respiratory burst is performed by a multicomponent nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase and is critical for the bactericidal action of phagocytes (DeCoursey, 2010; El-Benna, 2008). NADPH oxidase are also involved in the pathogen defense mechanism in plants (Foyer, 2005; Torres, 2006).
In figure 10, a phagocyte is depicted engulfing a bacterium into an emerging phagosome, which will subsequently close. NADPH oxidase assembles preferentially in the phagosomal membrane in neutrophils and begins to function before the phagocytic cup has sealed. The entire system is driven by NADPH oxidase activity. Electrons from cytoplasmic NADPH are translocated across a redox chain to reduce $O_2$ to $O_2^{-}$ inside the phagosome or extracellularly. For each electron removed from the cell, approximately one proton is left behind. Thus NADPH oxidase activity tends to depolarize the membrane, decrease the pH of the cytoplasm, and increase the pH of the
extracellular environment or the pH of the phagosome. NADPH is regenerated continuously by the hexose monophosphate shunt (HMS) during the respiratory burst. The superoxide produced can attack the bacteria inside the phagosome directly or it may be converted in alternate reactive oxygen species like H$_2$O$_2$, ONOO$^-$ and HOCl which will be used to defend against the pathogen (DeCoursey, 2010, Murphy, 2006).

The respiratory burst presents an excellent example of how organisms have adapted precise mechanisms of control over ROS that has allowed them to use these potentially harmful molecules to their advantage. It shows that ROS are not only a nuisance that aerobic life must deal with but also a vast family of products that have been integrated in the normal function of cells.

1.6. ROS stress and oxidative damage

Owing to the highly reactive nature of ROS, there is a fine line between the beneficial use of ROS and the detrimental effects they can have on their substrates. All living organisms go to great lengths in order to control their extracellular and intracellular environments and keep the redox state at a homeostatic level. In healthy aerobes, the production of reactive species is approximately balanced with antioxidant defense systems. However the balance is not perfect and this leads to continuous damage to the cell. Examples, like in *E.coli*, where peroxide levels are maintained at 0.2μM
shows that antioxidant defenses control ROS rather than eliminating them (Lushchak, 2001). This has been attributed to the fact that the energy cost of antioxidant production is more than that of the repair systems that are in place to replace damaged biomolecules. In addition, as previously mentioned, ROS are also involved in the normal functioning of the cell. Finally, some ROS like the hydroxyl radical are just too reactive and the damage is done before the appropriate antioxidant can detoxify it (Halliwell, 2007).

The term ‘oxidative stress’ refers to a severe imbalance between ROS production and antioxidant defenses. In the words of Helmut Sies, oxidative stress is defined as “a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage” (Sies, 1997). Such oxidative damage refers to the biomolecular disruptions caused by the attack of ROS on the constituents of the living organism. Increased levels of oxidative damage is not a clear reflection of the levels of oxidative stress but rather can be brought upon by the failure of repair or replacement systems (Sies, 1997).

1.7. Defining antioxidants

Aerobic organisms survive the poisonous effects of oxygen simply because they contain antioxidant defense systems. A cell’s antioxidants can be synthesized de novo or can be acquired from dietary sources. The term antioxidant is widely used in literature and its specific definition is up for
discussion since the hierarchy of antioxidant capacity depends to some extent on the parameters within which it is used. In simplest term, an antioxidant system is any substance or actions that delays, prevents or removes oxidative damage to a target molecule. There is no universal 'best' antioxidant and the rank depends on the nature of the oxidative challenge. One of the simplest antioxidant defenses would be to minimize exposure to oxygen (Halliwell, 2007).

1.7.1. Enzyme-based antioxidant defense systems in bacteria

Faced with the constant threat of ROS in an oxygen-rich atmosphere, microbes have a vast repertoire of enzymes which they call upon to eliminate as much of the stress as possible. These proteins essentially maintain redox homeostasis by mechanisms that are based either on the redox properties of metalloproteins (NADPH independent) or on the reduction of NADPH.

1.7.1.1. The superoxide dismutase (SOD) family

As the name suggests, the SOD enzyme catalyses the metal-dependent dismutation of O$_2^-$ to H$_2$O$_2$ (Figure 11). SOD is the best-studied enzyme for combating the effects of oxidative stress and it is found in all aerobic organisms.
\[ 2 \text{O}_2^- + 2 \text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2 \]

**Figure 11.** SOD detoxifies the superoxide anion (Wang, 2006).

There are four classes of SOD which differ from each other in relation to the transition metals in their active site (Scandalios, 2005):

- Iron-dependent SOD (FeSOD)
- Manganese-dependent SOD (MnSOD)
- Copper-dependent SOD (Cu/ZnSOD)
- Nickel-dependent SOD (NiSOD)

The importance of these enzymes has been shown in various mutagenesis studies where the deletion of SOD genes in bacteria as well as in mice have had disastrous outcome on the organism's ability to function. A key feature of these studies was the hypersensitivity to oxygen and to superoxide generating chemicals like menadione or paraquat. These types of experiments revealed the important role of SOD in DNA protection due to the increase mutation frequency in the mutant organism (Lebovits, 1996; Purdy, 1994). This is likely due to the release of iron from [Fe-S] clusters of enzymes by O$_2^-$ reactivity. The free iron can then undergo Fenton and Haber-Weiss
reactions, thus producing the mutagenic hydroxyl radical (Miller, 2004).

Since spontaneous reduction of superoxide is unfavorable, SOD catalyzes the reaction in two steps. In the first step, SOD strongly accelerates the reduction of the radical anion by donating a proton. The energy released from the first step is then used in the second step. SOD has the largest $k_{cat}$ (reaction rate with its substrate) of any known enzyme ($\sim 7 \times 10^5$ M$^{-1}$s$^{-1}$). This reaction is limited only by the frequency of collision between itself and $O_2^-$ and therefore it is said that the reaction rate is "diffusion limited" (Moosavi-Movahedi, 2005).

1.7.1.2. Superoxide reductases

Until recently, SOD was the only enzyme known to eliminate superoxide but certain bacteria such as *Desulfovibrio baarsii*, *Acheoglobus fulgidus* and *Treponema pallidum* utilize another enzymatic system to rid themselves of the anionic radical. This enzyme is called superoxide reductase (SOR) (figure 12) (Lombard, 2000; Nivière, 2004).

\[
O_2^- + 2H^+ + 1e^- \xrightarrow{SOR} H_2O_2
\]

*Figure 12. SOR detoxifies the superoxide anion.*
SOR is a small metalloprotein with an iron-containing active site which is situated at the surface of the protein. Easily accessible to the solvent, the iron atom is ligated through a square pyramidal geometry. High spin Fe$^{2+}$ is coordinated with 4 planar histidine residues and an axial cysteine. The 6th position coordinating the iron is vacant which suggests this is an entry site of the ROS substrate. Once the reaction is completed the high spin Fe$^{3+}$ is coordinated with a conserved glutamate residing in the 6th position. During the catalytic cycle of SORs, the superoxide anion reacts with the Fe$^{2+}$ of the active site ($k$=1.109 M$^{-1}$s$^{-1}$). A positively charged proximal lysine residue favors the high rate constant by guiding the anion to the site of catalysis (Yeh, 2000). Several intermediates are formed in the process and the rate of the protonation events depend on the pH, which indicates that the proton comes directly from the solvent. Peroxide is finally released leaving an oxidized enzyme that has no specific preference for its regeneration and is capable of accepting electrons from a large spectrum of donors (Mathe, 2005).

Superoxide dismutase and superoxide reductase are both efficient defense mechanism against increasing superoxide levels, however, they do not relieve the cell completely from oxidative stress. These enzymes lead to the production of the non-radical ROS, H$_2$O$_2$, which must be detoxified by another set of enzymes.
1.7.1.3. The reduction of $\text{H}_2\text{O}_2$ by catalase

To cope with endogenous $\text{H}_2\text{O}_2$ produced by enzymatic activity as previously mentioned or to resist an onslaught of exogenous peroxide stress, cells evokes a family of enzymes called catalases. Most catalases are formed of 4 subunits of 60 or 75 kDa, with each subunit containing a heme-based active site embedded in the protein and accessible through hydrophobic channels. These enzymes catalyze the dismutation of hydrogen peroxide (figure 13) using the catalytic power of the iron-containing heme. In the first step of the reaction, $\text{H}_2\text{O}_2$ oxidizes the heme into a "oxyferryl" species (known as compound I) which is then reduced by a second $\text{H}_2\text{O}_2$ molecule in the later step, consequently regenerating the enzyme for further detoxification reactions. The net reaction of the catalases takes two $\text{H}_2\text{O}_2$ molecules and yields a water and molecular oxygen. Excess levels of $\text{H}_2\text{O}_2$ may promote the formation of an intermediate compound II ($\text{Fe}^{IV}$OH) due to one electron oxidation; this intermediate is not active in $\text{H}_2\text{O}_2$ removal. NADPH binding is important to maintain an active enzyme as such binding can prevent the formation of compound II (Kirkman, 2006; Wang 2006).

\[
\text{Enz–Fe(III)} + \text{H}_2\text{O}_2 \rightarrow \text{Enz}^{\circ}\text{Fe(IV)=O} + \text{H}_2\text{O}
\]

\[
\text{Enz}^{\circ}\text{Fe(IV)=O} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{Enz–Fe(III)} + \text{H}_2\text{O}
\]

**Figure 13.** The two-step reduction of hydrogen peroxide by catalases.
Catalases were one of the first enzymes described in *E. coli*, which possesses two types of catalases, HPI (a homotetramer) and HP II (a homohexamer) encoded by the genes KatG and KatE respectively (Chan, 2010). In other bacteria such as *Thermus thermophilus* and *Lactobacillus plantarum*, catalases with binuclear manganese active sites have been identified (Antonyuk, 2000). Catalase is a well-studied ubiquitous enzyme that plays an important role in antioxidant defense. They are, however, not the only hydrogen peroxide scavenging antioxidants.

### 1.7.1.4. Thiol-dependent peroxidases

Thiol-dependent peroxidases are relatively small non-heme proteins capable of catalyzing the one electron reduction of peroxides (ROOH) into their corresponding alcohols (ROH). Their catalytic activity relies on the presence of a reactive cysteine which can act as a nucleophile and attack the peroxide functional group. This reaction yield a water molecule or an alcohol and the formation of a sulfinic acid (Cys-OH) in the active site. The reactivity of the active site cysteine is possible when it is in its more potent nucleophilic thiolate form (Cys-S-) rather then its protonated form. For this reason, all thiol-dependent peroxidases have a conserved arginine residue in their active site, which allows to lower the pKa of the cysteine and favor the thiolate form of the activated amino acid (Dubbs, 2007; Poole, 2003).
There are two main classes of peroxidases that have been described: 
the peroxiredoxins and the glutathione peroxidases. The non-heme 
glutathione peroxidases catalyze the rapid reduction of hydrogen peroxide 
and organic hydroperoxides, using a covalently-incorporated selenium at the 
active site as part of a redox-active selenocysteine residue (Figure 14) (Ursini, 
1995).

![Figure 14. Gluthathione peroxidase reaction with hydrogen peroxide.](image)

Alternatively, there are three classes of peroxiredoxins (Prxs) that are 
distinguishable by features of their catalytic sites. By far the most prevalent, 
abundant and active Prxs in bacteria are the close relatives of the "typical" 2-Cys Prxs. As mentioned above, these enzymes possess no metal or 
prosthetic group, but rely on a single peroxidatic cysteiny1 residue for 
hydroperoxide reduction and a second cysteine for progression of the 
catalytic cycle to intersubunit disulfide bond formation (Figure 15). For attack 
on the – O-O– bond of the hydroperoxide (ROOH, including the peroxynitrite),
the strictly conserved peroxidatic cysteine is activated through electrostatic interaction with a conserved arginine residue and hydrogen bonding to a conserved threonine, lowering it pKa. Following attack by the thiolate anion, the first product, is likely protonated and released, and the catalytic cysteine is oxidized to a cysteine sulfenic acid (Cys-SOH).

![Figure 15. Catalytic cycle of 2-Cys peroxiredoxins.](image)

As the figure above demonstrates, once formed, the cysteine thiol disulfide bonds are reduced by other proteins. An example of these proteins are thioredoxins (denoted “CXXC” protein(s) in figure 15.). Thioredoxins are small oxidoreductase enzyme containing a dithiol-disulfide active site. They are ubiquitous and found in many organisms from plants and bacteria to mammals. Thioredoxins are characterized at the level of their amino acid sequence by the presence of two vicinal cysteines in a CXXC motif. These two cysteines are the key to the ability of thioredoxin to reduce other proteins. The thioredoxins are kept in the reduced state by the flavoenzyme thioredoxin reductase, in a NADPH-dependent reaction. Thioredoxins act as electron
donors to peroxidases (Poole, 2003; Wang, 2006)

1.7.2. Low molecular weight antioxidants

Organisms under oxidative insult evoke an arsenal of defense mechanisms allowing them to survive the harsh environment and return to a homeostatic redox level. The repertoire of antioxidant defense is not limited to enzymes but also include a spectrum of low molecular weight molecules that scavenge ROS throughout the cells. These are known as low molecular weight antioxidants (LMWA). LMWA can react and deactivate free radicals and oxidants. These antioxidants vary in chemical structure and can be either water- or lipid-soluble. Microorganisms synthesize their own LMWA but higher eukaryotes often take up these molecules through their diet (Grune, 2005).

Vitamin C, also known as ascorbic acid (AA) (figure 16), is a water-soluble vitamin that is an essential cofactor for several enzymes including proline hydroxylase and lysine hydroxylase. In humans, a deficiency in ascorbate is characterized by unstable collagen due to the insufficient hydroxylation of collagen molecules which can lead to scurvy disease. AA is one of the most extensively studied antioxidants and is a strong reducing agent that can react with $\text{H}_2\text{O}_2$, $\text{O}_2^-$, 'OH and lipid hydroperoxidases. Several nitroso-compounds are also reduced by AA and this LMWA plays an additional role in protecting or regenerating oxidized carotenoids or
tocopherols. Ascorbate will react with a free radical leading to the production of the unstable ascorbyl radical. Oxidized ascorbate can be recycled by NAD(P)H- or GSH-mediated enzymatic activities. AA minimizes the damage caused by oxidative process through synergic function with other antioxidants (Gruner, 2005; Shao, 2008).

Vitamin E is one of the most important lipid-soluble chain-breaking antioxidant. The term ‘Vitamin E’ refers to a group of substances able to interfere with lipid peroxidation. All these compounds contain the chromanol ring with several hydrogen or methyl substituents in the positions C5, C7, or C8. This results in the so-called α-, β-, γ-, or δ-derivatives. The side chain may be either saturated or contain three unsaturated bonds. This divides the compounds into the tocopherols or the tocotrienols (figure 16). Tocopherols are lipophilic antioxidants that interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various reactive oxygen species (ROS) and lipid soluble byproducts of oxidative stress. Singlet oxygen quenching by tocopherols is highly efficient, and it is estimated that a single α-tocopherol molecule can neutralize up to 120 singlet oxygen molecules in vitro before being degraded.
Ascorbic acid  \( \alpha \)-Tocopherol  
\[
\begin{align*}
\text{HO} & \quad \text{HO} \\
\text{HO} & \quad \text{HO} \\
\text{\textbullet} & \quad \text{\textbullet}
\end{align*}
\]
\[
\begin{align*}
\text{HO} & \quad \text{HO} \\
\text{HO} & \quad \text{HO} \\
\text{\textbullet} & \quad \text{\textbullet}
\end{align*}
\]

\( \alpha \)-Tocotrienol

\[
\begin{align*}
\text{HO} & \quad \text{HO} \\
\text{HO} & \quad \text{HO} \\
\text{\textbullet} & \quad \text{\textbullet}
\end{align*}
\]

**Figure 16.** Chemical structures of ascorbate and vitamin E (Grune, 2005).

Because of their chromanol ring structure, tocopherols are capable of donating a single electron to form the resonance-stabilized tocopheroxyl radical. \( \alpha \)-Tocopherols also function as recyclable chain reaction terminators of polyunsaturated fatty acid (PUFA) radicals generated by lipid oxidation. \( \alpha \)-Tocopherols scavenge lipid peroxy radicals and yield a tocopheroxyl radical that can be recycled back to the corresponding \( \alpha \)-tocopherol by reacting with ascorbate or other antioxidants (Grune, 2005; Shao, 2008).

### 1.7.3. Gene regulation involved in ROS detoxification

It is one thing to have a repertoire of ROS detoxifying enzymes and be able to synthesize LMWA, but organisms must possess mechanisms to tailor
the use of these antioxidants systems to a certain insult. In other words, cells must be able to recognize when the burden of ROS becomes excessive and that they are under oxidative stress. They must then be able to regulate the mobilization of the proper antioxidant defense mechanisms and down-regulate their pro-oxidant generating pathways. Genetic response to oxidative stress occurs in all aerobic organism including bacteria, yeast, plant and mammalian cells. Microbes like E.coli possess a specific defense against peroxide which is mediated by the transcriptional activator OxyR along with another against superoxide, controlled by the two-staged SoxRS system. The activation of these response elements greatly increases oxidative stress resistance (Ros, 2000).

1.7.3.1 OxyR - a sensor for hydrogen peroxide

The OxyR protein plays the role of a sensitive sensor in a response to an increase in H₂O₂ levels in a cell. The protein exists in two forms, oxidized and reduced. Direct oxidation of OxyR is responsible for the activation of transcription. Both OxyR forms bind DNA, but their binding specificity is different. The reduced form can bind oxyR and mom promoters but not the katG (catalase) and ahpC (peroxiredoxin) promoters. Oxidized OxyR has been found to bind all OxyR-regulated promoters. Differences in binding specificity may allow OxyR to carry out a variety of functions according to the conditions faced by the cell. Therefore, OxyR can repress the oxyR and mom
promoters during normal growth and activate *katG* and *ahpC* in response to oxidative stress. An increase in H$_2$O$_2$ induces the oxidation of the OxyR regulon by the sequential oxidation of Cys-199 residue followed by Cys-208 residue resulting in the formation of an intramolecular disulfide bond (figure 17). The formation of this disulfide bond induces conformational changes thus activating the transcription factor.

**Figure 17.** OxyR regulation (Modified from Ros, 2000).
OxyR is deactivated by reduction by glutaredoxin (Grx) which together with AhpC and catalase, contribute to OxyR auto-regulation. As these enzymes, regulated by OxyR, detoxify the H₂O₂ they cause the deactivation of the OxyR and the return to redox homeostasis (D’Autréaux, 2007; Dubbs, 2007; Poole, 2004; Ros, 2000).

1.7.3.2. SoxRS - a sensor for the superoxide anion

The SoxR transcription factor is an O₂⁻- specific sensor that also plays a small role in response to reactive nitrogen species (RNS). This transcription factor demonstrates its specificity for superoxide with its target genes, whose products are involved in O₂⁻- catabolism (for example SOD) and protection or repair of [Fe-S] clusters (for example oxidation-resistant [2Fe-2S] containing fumarase C and [Fe-S]-repair ferredoxin: NADPH oxidoreductase). Oxidation of the SoxR [2Fe-2S] cluster by superoxide cause conformational changes in the transcription factor’s structure which allows DNA binding and gene activation. SoxR binds to DNA at a specific site and promotes transcription of the soxS gene, resulting in an increase of SoxS protein (Figure 18). It is this protein which activates genes that are involved in the response to superoxide insult such as endonuclease IV and glucose-6-phosphate dehydrogenase (NADPH-dependent) (D’Autréaux, 2007; Lushchak, 2001).
Gene activation affording cellular resistance to stress induced by superoxide.

Figure 18. SoxRS regulation (Modified from Lushchak, 2001).

1.7.4. Oxidative damage and repair mechanisms

As efficient as cells are at dealing with oxidative stress, damage still occurs. An important part of the antioxidant defense mechanism includes
repair mechanisms for the ROS-produced injuries. In the case of lipids, peroxidases participate in the decomposition of lipid peroxides and lipases are responsible for hydrolyzing damaged fatty-acid chains. In another instance, apart from a few select enzymes such as aconitase, not much information is available about protein repair mechanisms. However, as previously mentioned, mildly oxidized proteins are hydrolyzed more rapidly than native ones by proteasomes (Grune, 2003).

One of the most extensively studied areas of protection against ROS through repair mechanisms is the restoration of damaged DNA molecules. This aspect of antioxidant defense is very important for the survival of injured cells. The processes of DNA damage can be divided into two stages: the formation of primary products that are mainly unstable, and the formation of end products which are formed as a result of hydrolysis and reorganization of the primary products (Lushchak 2001).

The types of DNA damage are separated into damage to nitrogen-containing bases and damage to deoxyribose. DNA glycosylases initiate the repair by hydrolyzing bonds between the base and carbohydrate of a modified or "incorrect" nucleotide and result in formation of abasic areas. Formamidopyrimidine glycosylase releases fragmented purine lesions (formamidopyrimidines, or FAPy) from methylated, alkali-treated DNA. The
protein MutY is also a part of the defense system against mutagenesis in certain bacteria. It efficiently removes adenine from 8-oxoG-containing DNA base pairs. *E. coli* has an exonuclease III which has a preference for double-stranded substrates and an endonuclease IV whose activity is induced 10-fold in response to certain superoxide-generating agents or by nitric oxide. These enzymes are complemented with appropriate helicases, polymerases, and ligases that eliminate damaged oligonucleotides and terminate the repair process (Lushchak, 2001).

1.8. Metabolism and the new perspectives on antioxidant defense

All previously mentioned antioxidant systems are "classical" mediators that work in concert with each other to enable cells to live in a toxic oxygenated environment. However, a new tool is beginning to emerge as a significant antioxidant defense mechanism. Organisms threatened by oxidative stress undergo extensive metabolic shifts. These modulations of catabolic and anabolic pathways allow for the production of key players in the fight against ROS and limit the production of pro-oxidants by the cell. Going back to the definition of an antioxidant system as "any substance or actions that delays, prevents or removes oxidative damage to a target molecule", it is evident that the reprogramming of metabolic pathways represents a prime example of antioxidant action.
1.8.1. ROS-detoxifying systems increase the NADPH demand of an organism

Many ROS-scavenging systems such as peroxiredoxins, glutathione reductase (GR) and ascorbate require the need of NADPH to reduce and regenerate their oxidized active sites. Others, like catalase, use NADPH to avoid the formation of intermediates that hinder the capacity of the enzyme to do its job (Kirkman, 2006). Regardless where it is used, NADPH is the conductor orchestrating the cell’s antioxidant concert. For this reason, NADPH-generating enzymes are highly active under oxidative stress conditions in order to maintain the reducing power needed by the cell. These enzymes include glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), NADP-dependent isocitrate dehydrogenase (NADP-ICDH) and NADP glutamate dehydrogenase (NADP-GDH). They are involved in central metabolism, are employed by the cell to produce NADPH and keep the cytosolic compartment of the cell in a reduced environment (Mailloux, 2011).

Studies centered on endogenous metabolic oxidative stress have uncovered the function of various cellular antioxidants and the importance of NADPH as the ultimate reductive force required for ROS detoxification. For example, mutant yeast lacking SOD and catalase were more susceptible to oxygen toxicity. In addition to SOD and catalase gene knockouts, the effect of co-disruption for the gene zwf1 (encoding G6PDH) and idp2 (encoding NADP-
ICDH) yielded yeast cells unable to grow on oleate as a carbon source. The detrimental effect on cell viability is likely due to the loss of the these two cytosolic sources of NADPH. When growing on fatty acids, high levels of H₂O₂ produced by β-oxidation would require NADPH-mediated detoxification by glutathione- and thioredoxin-dependent peroxidases. Consequently, since the NADPH sources are depleted, the yeast cannot grow (Minard, 2001).

Further evidence to support the role of metabolic shifts has been reported by Mailloux et al (Mailloux 2011). Studying the soil microbe Pseudomonas fluorescens during oxidative insult, the group has identified a reconfiguration of metabolic processes to meet the need for NADPH. Under oxidative conditions, metabolism shifts in the favor of NADPH production and away from NADH generation. The key to shifting the balance from NADH to NADPH production is the upregulation of NAD kinase and the down-regulation of NADPase (figure 19) (Mailloux, 2011).
Figure 19. Metabolic switch leading to the increase production of NADP and NADPH (Mailloux, 2011).

Along with the increase in levels of the substrate NADP$^+$ through NADK, *P. fluorescens* evokes an efficient NADH to NADPH conversion cycle in order to couple the enhanced NADPH production with the diminished synthesis of NADH. This cycle is made possible by the reconfiguration of enzymes involved in central metabolic pathways such as the tricarboxylic acid (TCA) cycle, glycolysis and gluconeogenesis: malate dehydrogenase (MDH), malic enzyme (ME), pyruvate carboxylase (PC) and pyruvate kinase (PK) converge to form the NADPH producing machinery of the conversion cycle (figure 20) (Singh 2008).
Figure 20. NADH/NADPH conversion cycle (Modified from Singh, 2008).

Although the modulation of the production of NADPH is instrumental for the regeneration of other antioxidant systems, the decrease in NADH levels also enhances the cell’s defense against ROS. Since NADH is the electron donor to the ETC, a decrease level of this substrate would force the cell to limit the usage of this ATP-producing machinery. At first glance, this action
may seem at odds with the promotion of cell survival, but when the endogenous production of ROS by the ETC is taken in consideration, it is a wise move by the cells to shut down oxidative phosphorylation. This shift away from cellular respiration has been observed in *P. fluorescens* and numerous organisms exposed to oxidative stress (Mailloux, 2011).

1.8.2. α-Ketoacids as antioxidants

Even though a majority of the attention is given to enzyme-based antioxidants and DNA repair systems, an increasing body of literature is revealing the role of α-ketoacids in ROS detoxification. α-Ketoacids are organic compounds that contain a carboxylic acid group adjacent to a ketone group. They play an essential role in cellular metabolism as intermediates in many pathways including the TCA cycle and glycolysis. Examples of biologically relevant α-ketoacids include pyruvate, α-ketoglutarate, oxaloacetate and glyoxylate. The antioxidant potential of these substrates has been demonstrated in a variety of ways.

Numerous studies have shown the benefits of α-ketoacid supplementation in order to prevent or rectify oxidative damage. An area of this research includes the prevention of cataract formation by pyruvate. Cataracts are characterized by the clouding that develops in the crystalline lens of the eye, which can vary from a slight degree of opacity to the
obstruction of the passage of light. Cataract formation has been linked to diabetes, hypertension and the over-exposure to UV-radiation. Although treated with surgery and replacement with synthetic implants there is a push for the development of pharmacological means of cataract prevention, which would reduce invasiveness and cause less secondary effects. The UV-radiation hypothesis of cataract formation states that photons penetrate through to cornea and cause subsequent induction of photochemically generated ROS in the aqueous humor and lens of the eye. Accordingly, cataract formation is associated with many signs of oxidative stress such as excessive protein glycation and lipid peroxidation, depletion of glutathione and a decrease in ATP levels. In vitro incubation of mice and rat eye lens with pyruvate demonstrated the beneficial effect of this α-ketoacid in preventing cataract formation by scavenging ROS. These studies demonstrate therapeutic potential of pyruvate in offsetting the cataractogenesis effects of solar radiation and other factors that act via ROS toxicity (Hegde, 2007, Hegde, 2005).

The use of α-ketoacids in the detoxification of ROS has been shown in many other cases. In numerous brain pathologies such as neurodegenerative disease or in acute injuries such as ischemia or trauma, H₂O₂ is a suspected culprit in disease development. A study by Desagher et al. (1997) examined the ability of pyruvate to improve the survival of cultured striatal neurons.
exposed to $H_2O_2$. Surely enough pyruvate protected neurons against both $H_2O_2$ added to the external medium and $H_2O_2$ endogenously produced through the redox cycling agent menadione. The neuroprotective effect of pyruvate appeared to result from the ability of the $\alpha$-ketoacid to undergo non-enzymatic decarboxylation in the presence of $H_2O_2$ (figure 21). In addition, several other $\alpha$-ketoacids including $\alpha$-ketobutyrate, which is not an energy substrate, also provided the neuroprotective effect of pyruvate. This study also showed that optimal neuroprotection was achieved with relatively low concentrations of pyruvate (>1mM) and that due to its low toxicity and its capacity to cross the blood-brain barrier, this $\alpha$-ketoacid opens a new therapeutic perspective in ROS associated-brain pathologies (Desaghe, 1997).

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CH₃-C-*C + H₂O₂ → CH₃-C + *CO₂ + H₂O

pyruvate acetate
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**Figure 21.** Non-enzymatic decarboxylation of the $\alpha$-ketoacid pyruvate.

The role of pyruvate as a physiological antioxidant was further confirmed by an interesting observation. Biagini *et al.* (2001) studied the metabolism of the two parasitic diplomonads *Giardia intestinalis* and *Hexamita inflata*, which
lack the ability to perform oxidative phosphorylation and therefore rely on fermentative metabolism. When supplemented with exogenous pyruvate, levels of ROS were shown to decrease in the diplomonads. In contrast, the aerobic yeast *Saccharomyces cerevisiae* showed an increase in the generation of oxidative species following addition of pyruvate, presumably because the metabolism of the ketoacid increased electron leakage from the ETC. These data suggest that in *G. intestinalis* and *H. inflata*, pyruvate exerts antioxidant activity at physiological levels. More importantly, it is the absence of a respiratory chain in the diplomonads which facilitates the observed antioxidant activity. This fact is important when considering metabolic shifts. Other studies have shown the link between oxidative phosphorylation and ROS production thus resulting in a limited use of the ETC during oxidative insult (Biagini, 2001).

The use of these antioxidants, which are often found at the crossroad of metabolic pathways, has also been shown to be induced through metabolic shifts in-vivo. Metabolic shifts leading to the accumulation of α-ketoacids within a cell have been observed in *P. fluorescens* and human hepatocytes. Cells grown on citrate and supplemented with the different amino acids glutamate, proline, and arginine as nitrogen sources all demonstrated a similar metabolic reconfiguration under menadione-induced oxidative stress. First, the TCA cycle is modulated by the decrease activity of α-ketoglutarate
dehydrogenase. Furthermore, NADP-dependent glutamate dehydrogenase is highly active in order to assure α-ketoglutarate (α-KG) accumulation. This keto acid can then react with ROS, giving succinate as a product, thus bypassing two steps of the TCA cycle while detoxifying the stress. This strategy (represented in figure 22) offers an effective way of eliminating ROS and does not require the direct or indirect utilization of NADPH. Compared to other detoxifying methods such as GSH, metabolic reconfiguration for the accumulation of α-KG may be more advantageous. The GSH tripeptide has to be regenerated, a process that is mediated by NADPH and GSH reductase and its synthesis requires three amino acids and ATP, factors which may become limited during oxidative stress (Lemire, 2010a,b; Singh, 2009a).
Figure 22. Metabolic shifts in the TCA cycle lead to the accumulation of ketoacids for the detoxification of ROS (Singh, 2009a).

In hepatocytes, the generation of succinate as a consequence of the ROS detoxifying activity of α-KG provides a signaling metabolite promoting anaerobiosis. Under certain conditions like oxidative stress or low oxygen levels, mammalian cells invoke a hypoxic response which enables the production of ATP through substrate level phosphorylation. This adaptive response is initiated by the heterodimeric transcription factor HIF-1. HIF-1
consists of HIF-1α, HIF-2α and HIF-1β subunits. HIF-1α is extremely sensitive to oxygen tension and, under normoxic conditions, HIF-1α is quickly degraded by prolyl hydroxylases (PHDs) and the ubiquitin-proteosome degradation pathway. Prolyl hydroxylation demarcates HIF-1α for degradation by the proteosome. PHD induces HIF-1α degradation by the hydroxylation of key proline residues in the oxygen-dependent degradation domain, a catalytic process which requires α-KG, Fe, and O₂. Three separate PHD isozymes (PHD1-3) have been implicated in this process with PHD2 being the most characterized. Hypoxia, Fe limitation, and oxidative stress appear to impede PHD activity thus stabilizing HIF-1α. These events promote the activation of the hypoxic gene programs which encourage cell survival, glucose utilization, substrate level phosphorylation, erythropoiesis, and glycolysis. The activation of glycolysis during hypoxia is crucial to cell survival since it allows energy production without the participation of the ETC. Succinate is known to perturb substrate binding sites in PDH2 thus interfering with the degradation of HIF-1α and in turn promoting anaerobic metabolism (figure 23) (Mailloux, 2009; Mailloux, 2006).
Figure 23. Succinate accumulation signals anaerobiosis.
Thesis objectives

Oxidative stress is a constant threat to aerobic life. The various sources of ROS in the environment have compelled organisms to evolve elaborate antioxidant defense mechanisms. Living systems utilize enzymes such as superoxide dismutase (SOD) and catalase along with scavengers like glutathione (GSH) and α-tocopherol to protect themselves against reactive oxygen species (ROS). Recently, our laboratory has unraveled the roles of metabolic networks and α-ketoacids in neutralizing ROS. α-Ketoacids usually represent central metabolism substrates which have been shown in a variety of organisms to readily accumulate during oxidative stress. They interact with ROS to liberate CO₂ and the corresponding acids.

In cultured hepatocytes, Mailloux et al. (2011) have demonstrated the intricate metabolic shifts leading to the pooling of α-KG and the production of NADPH which enables the cells to resist the ROS-toxicity induced by aluminum stress. In this instance, the ROS-derived succinate acts as a potent signaling metabolite aimed at promoting anaerobiosis. The role of α-KG was further examined in the soil microbe *P. fluorescens* grown on citrate as a carbon source and exposed to exogenous H₂O₂ and the redox-cycling agent menadione (Mailloux, 2011). This microbe is an ideal model system to decipher metabolic networks due to its nutritional versatility and its ability to proliferate in various environments. This gram-negative bacterium is resilient
and extremely adaptive to diverse environmental conditions such as metal- and oxidatively-stressed milieu. Hence, it is not surprising that *P. fluorescens* is widely utilized in numerous biotechnological processes.

As *P. fluorescens* readily modifies its metabolic pathways to generate α-KG, an antioxidant, it is important to delineate if other ketoacids are also involved in the ROS detoxification strategy. In the current work, the microbe is cultured in various glycolytic amenable carbon sources like glucose, fructose, xylose, sucrose and glycerol in the presence of elevated levels of H$_2$O$_2$ and O$_2^-$ (menadione) respectively in an effort to assess the contribution of pyruvate as a ROS scavenger. It is expected that since these carbon sources would transit via this keto-acid, the organism would alter its metabolic networks to pool pyruvate. Furthermore, the acetate generated as a consequence of the ROS-mediated pyruvate decomposition, would lead to a unique metabolic profile in the microbe exposed to oxidative stress.

Although during oxidative stress the production of energy is severely affected, there is a dearth of information in the literature pertaining to the strategies living systems invoke to fulfill their need in ATP. In this study, the biochemical pathways *P. fluorescens* cells utilize to achieve ATP homeostasis has been investigated. As oxidative phosphorylation is perturbed by ROS, the influence of substrate level phosphorylation and phosphotransfer systems in
aiding the biosynthesis of ATP have been assessed. Metabolomic and proteomic approaches are utilized to probe the homeostasis of pyruvate, an antioxidant, and ATP in *P. fluorescens* grown in an oxidative environment in an effort to unravel other facets of anti-oxidative defense mechanisms, which are only beginning to emerge.
2. Materials and Methods

2.1. List of reagents and equipment

2-Thiobarbituric acid; Sigma-Aldrich (St. Louis, Missouri)

2,4-Dinitrophenylhydrazine; ICN Biochemicals (Cleveland, Ohio)

2,6-Dichloroindophenol; Sigma-Aldrich (St. Louis, Missouri)

6-aminohexanoic acid; Sigma-Aldrich (St. Louis, Missouri)

6-phosphogluconic acid; Sigma-Aldrich (St. Louis, Missouri)

Accumet pH meter 910; Fisher Scientific (Unionville, Ontario)

Acrylamide; Bio-Rad Laboratories (Mississauga, Ontario)

Acetyl coenzyme A; Sigma-Aldrich (St. Louis, Missouri)

Adenosine 5' triphosphate (ATP); Sigma-Aldrich (St. Louis, Missouri)

Adenosine 5' diphosphate (ADP); Sigma-Aldrich (St. Louis, Missouri)

Adenosine 5' monophosphate (AMP); Sigma-Aldrich (St. Louis, Missouri)

α-Ketoglutaric acid; ICN Biochemicals (Cleveland, Ohio)

Alliance 2487 Dual Wavelength Absorbance Detector; Waters Ltd. (Mississauga, Ontario)

Alliance 2695 separation module, High performance liquid chromatographer; Waters Ltd. (Mississauga, Ontario)

Ammonium chloride; Sigma-Aldrich (St. Louis, Missouri)

Ammonium molybdate; Sigma-Aldrich (St. Louis, Missouri)

Ammonium persulfate (APS); Bio-Rad Laboratories (Mississauga, Ontario)

Bacto-Agar; Difco Laboratories (Detroit, Michigan)
Bio-Rad Mini-Protein II Dual Slab Cell; Bio-Rad Laboratories (Mississauga, Ontario)

Bis-acrylamide; Bio-Rad Laboratories (Mississauga, Ontario)

Bistris; Fisher Scientific (Unionville, Ontario)

Bovine serum albumin (BSA); Sigma-Aldrich (St. Louis, Missouri)

Bradford reagent; Bio-Rad Laboratories (Mississauga, Ontario)

Bromophenol blue; Fisher Scientific (Unionville, Ontario)

Bromo-pyruvate; Sigma-Aldrich (St. Louis, Missouri)

C18-reverse phase column with polar end cap; Phenomenex (Torrence, California)

Centrifuge Model J2-MI; Beckman Instruments (Mississauga, Ontario)

Citric acid monohydrate; Sigma-Aldrich (St. Louis, Missouri)

Coenzyme A (sodium salt); Sigma-Aldrich (St. Louis, Missouri)

Cytochrome C; Sigma-Aldrich (St. Louis, Missouri)

Coomassie Blue G-250; Sigma-Aldrich (St. Louis, Missouri)

Coomassie Blue R-250; Sigma-Aldrich (St. Louis, Missouri)

Diaminobenzidine; Sigma-Aldrich (St. Louis, Missouri)

Dithiothreitol (DTT); Sigma-Aldrich (St. Louis, Missouri)

D-glucose; Sigma-Aldrich (St. Louis, Missouri)

D,L-isocitric acid trisodium salt; Sigma-Aldrich (St. Louis, Missouri)

Ferric chloride; Fisher Scientific (Unionville, Ontario)

Fructose-6-phosphate (F6P); Sigma-Aldrich (St. Louis, Missouri)

Fumaric acid; Fisher Scientific (Unionville, Ontario)
Glacial acetic acid; CanLab (Toronto, Ontario)

Glucose-6-phosphate (disodium salt); Sigma-Aldrich (St. Louis, Missouri)

Glucose-6-phosphate dehydrogenase (from porcine heart); Sigma-Aldrich (St. Louis, Missouri)

Glycerol; Sigma-Aldrich (St. Louis, Missouri)

Glycine; Sigma-Aldrich (St. Louis, Missouri)

Gradient Former; Bio-Rad Laboratories (Mississauga, Ontario)

Guanidine-HCl; Sigma-Aldrich (St. Louis, Missouri)

Gyratory waterbath shaker model G-76; New Brunswick Scientific (Edison, New Jersey)

Hexokinase (HK) from porcine heart; Sigma-Aldrich (St. Louis, Missouri)

Hydrochloric acid ; CanLab (Toronto, Ontario)

Hydrogen peroxide (30% (w/v)); Sigma-Aldrich (St. Louis, Missouri)

Iodonitrotetrazolium chloride; Sigma-Aldrich (St. Louis, Missouri)

Lactate dehydrogenase (LDH) porcine heart; Sigma-Aldrich (St. Louis, Missouri)

Magnesium chloride tetrahydrate; Sigma-Aldrich (St. Louis, Missouri)

Malic acid; Sigma-Aldrich (St. Louis, Missouri)

Malate dehydrogenase (from porcine heart); Sigma-Aldrich (St. Louis, Missouri)

Menadione (sodium bisulfite); Sigma-Aldrich (St. Louis, Missouri)

β-mercaptoethanol; Sigma-Aldrich (St. Louis, Missouri)

n-Dodecyl β-D-maltoside; Sigma-Aldrich (St. Louis, Missouri)

Nicotinamide adenine dinucleotide (oxidized form); Sigma-Aldrich (St. Louis, Missouri)
Nicotinamide adenine dinucleotide (reduced form); Sigma-Aldrich (St. Louis, Missouri)

Nicotinamide adenine dinucleotide phosphate (oxidized form); Sigma-Aldrich (St. Louis, Missouri)

Nicotinamide adenine dinucleotide phosphate (reduced form); Sigma-Aldrich (St. Louis, Missouri)

Nitric acid; CanLab (Toronto, Ontario)

N,N,N',N'-Tetramethylenediamine (TEMED); Bio-Rad Laboratories (Mississauga, Ontario)

Oxythiamine; Sigma-Aldrich (St. Louis, Missouri)

P-anisidine; Sigma-Aldrich (St. Louis, Missouri)

Perchloric acid; CanLab (Toronto, Ontario)

Peristaltic pump; Fisher Scientific (Unionville, Ontario)

Phenazine methosulphate; Sigma-Aldrich (St. Louis, Missouri)

Phenol; Sigma-Aldrich (St. Louis, Missouri)

Phenylmethylsulphonylfluoride (PMSF); Sigma-Aldrich (St. Louis, Missouri)

Phosphoenolpyruvate (PEP); Sigma-Aldrich (St. Louis, Missouri)

Potassium cyanide; Sigma-Aldrich (St. Louis, Missouri)

Potassium phosphate monobasic; Sigma-Aldrich (St. Louis, Missouri)

Potassium phosphate monobasic, HPLC grade; Sigma-Aldrich (St. Louis, Missouri)

*Pseudomonas fluorescens* ATCC 13525; American Type Culture Collection (Rockville, Maryland)

Pyruvic acid; Sigma-Aldrich (St. Louis, Missouri)

Sodium bicarbonate; Sigma-Aldrich (St. Louis, Missouri)
Sodium phosphate dibasic; Sigma-Aldrich (St. Louis, Missouri)

Sodium hydroxide; Fisher Scientific (Unionville, Ontario)

Sodium dodecyl sulphate (SDS); Sigma-Aldrich (St. Louis, Missouri)

Spectrophotometer model Ultraspec 3000; Amersham Pharmacia Biotech (Baie d’Urfé, Québec)

Succinic acid; Sigma-Aldrich (St. Louis, Missouri)

Sulphuric acid; CanLab (Toronto, Ontario)

Trichloroacetic acid; Fisher Scientific (Unionville, Ontario)

Tricine; Sigma-Aldrich (St. Louis, Missouri)

Triethylamine; Sigma-Aldrich (St. Louis, Missouri)

Tris(hydroxymethyl)aminomethane-HCl and Tris(hydroxymethyl)aminomethane base; Sigma-Aldrich (St. Louis, Missouri)

Ultracentrifuge Model L8-M; Beckman Coulter, Inc. (Fullerton, Ontario)

Xylose; Sigma-Aldrich (St. Louis, Missouri)

### 2.2. Organism and growth conditions

The bacterial strain *Pseudomonas fluorescens* 13525 was obtained from the American Type Culture Collection (ATCC). A mineral medium containing citric acid solidified by 2% agar was used to keep the microbe. The sterile agar test tubes were maintained at 4°C. The bacteria were sub-cultured bi-weekly. Deionized and distilled water (ddH₂O) was utilized in all experiments.
2.2.1. Agar medium

A volume of 250mL of ddH₂O was used to dissolve Na₂HPO₄ (42.3mM); KH₂PO₄ (22.0mM); NH₄Cl (15mM); MgSO₄ (0.811mM); citric acid monohydrate (19mM) and 400μL of trace elements. The trace element solution consisted of FeCl₃•6H₂O (2μM); MgCl₂•4H₂O (1μM); Zn(NO₃)₂•6H₂O (0.05μM); CaCl₂ (1μM); CoSO₄•7H₂O (0.25μM); CuCl₂•2H₂O (0.1μM); NaMoO₄•2H₂O (0.1μM). To prevent the precipitation of the metals, the pH of the trace elements solution was adjusted to 2.75 with dilute HCl and the solution was stored at 4°C. The pH of the agar medium was raised to 6.8 with dilute NaOH and then the solution was slowly heated and Bactoagar (6.6g) was added and stirred until completely dissolved. The volume of the homogenous solution was brought up to 400mL with ddH₂O. Approximately 7 to 10mL of the solution were placed in test tubes and capped for slant. Following autoclave sterilization for 20min at 17lbs/in², 121°C, the test tubes were laid on an angle and allowed to solidify at room temperature. Slants were stored at 4°C.

2.2.2. Pre-culture medium

One litre of ddH₂O containing Na₂HPO₄ (42.3mM); KH₂PO₄ (22.0mM); NH₄Cl (15mM); MgSO₄ (0.811mM) and 1% (v/v) trace elements solution was adjusted to pH 6.8 with diluted HCl and separated into 100mL Erlenmeyer
flasks. The flasks were capped with foam plugs and autoclaved for 20 min at 17 lbs/in², 121°C. Once cooled, 7.5 mM glucose was added to the medium aseptically. It is important not to add the glucose prior to sterilization because the sugar will caramelize when heated. The pre-culture medium was inoculated with a loop of *P. fluorescens* stored on agar slants. Late-logarithmic phase of growth was attained following a 24-48 hrs incubation period in a gyratory water bath shaker model G76 (New Brunswick Scientific) at 26°C and 140 rpm. For some experiments the glucose was replaced with either 20 mM glycerol or 15 mM xylose as carbon sources.

2.2.3. Culture and cell growth

The medium used for liquid culture contained Na₂HPO₄ (42.3 mM); KH₂PO₄ (22.0 mM); NH₄Cl (15 mM); MgSO₄ (0.811 mM) and 1% (v/v) trace elements solution dissolved in ddH₂O. The pH of the solution was adjusted to pH 6.8 with diluted HCl and the volume was brought up to 1 L. The medium was then separated into 200 mL aliquots in 500 mL Erlenmeyer flasks. The flasks were capped with foam plugs and autoclaved for 20 min at 17 lbs/in², 121°C. Once cooled, 7.5 mM glucose was added to the medium aseptically. The carbon source was replaced with either 20 mM glycerol or 15 mM xylose for certain experiments. For the oxidative stress experiments, 100 μM H₂O₂ was added to the autoclaved media from an 8.88 M stock. For select experiments, the stressing agent was replaced for 100 μM menadione bisulfite.
from a 100mM stock. The media were then inoculated with 1mL of the pre-cultured bacteria. The cultures were incubated at 26°C in a gyratory water bath shaker model G76 (New Brunswick Scientific) at 140rpm. The medium without added H₂O₂ or menadione constituted the control medium. A second 100µM dose of H₂O₂ was added to the stress cultures after 15hrs of growth to ensure the cells were adequately stressed. To evaluate the dose tolerance towards H₂O₂, a 500µM dose of H₂O₂ was also used.

2.2.4. Harvesting of *P. fluorescens* and preparation of cell free extract (CFE)

*P. fluorescens* bacteria were collected from the growth medium at the late-logarithmic phase of growth by centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was removed and set aside for subsequent analysis. The bacterial pellet was washed and re-suspended in 0.85% NaCl. The bacteria were then centrifuged again at 10,000 x g for 15 min at 4°C and this procedure was repeated (Figure 24).
Figure 24. Harvesting cell of *P. fluorescens* cultures.

Following harvesting, the bacterial cells were re-suspended in a cell storage buffer (CSB) containing 50mM Tris-HCl, 5mM MgCl₂, 1mM PMSF, 1mM DTT at pH 7.3. The cells were disrupted by sonication (on ice) using a Brunswick sonicator, power level 4 for 15 sec for 4 intervals. Cells were stored on ice between intervals for approximately 3 min. The sonicated mixture, hereon referred to as the cell free extract (CFE), was collected and centrifuged at 180,000 x g for 180 min. The pellet (membrane fraction) was resuspended in cell storage buffer and stored at 4°C for immediate use or...
frozen in maltoside (10% maltoside) suspension for later use. The soluble fraction was removed and centrifuged a second time at 180,000 x g for 120 min to ensure a membrane free system. The resulting pellet was discarded as membranous cellular debris and the soluble fraction was either stored at 4°C for immediate use or frozen for later use. Samples of both fractions were kept at 4°C for a maximum of 3 days (Figure 25). For select experiments, once the bacteria from control and stress media were harvested, they were re-suspended in their respective opposite medium (control cells re-suspended in stress medium and vice-versa). After 8 hours of incubation in the new medium, cells were collected and processed as described above.
Figure 25. Isolation of CFE from whole cells.


**Figure 26.** Experimental design: Harvested cells were separated into their soluble and membrane CFE and metabolomics, proteomic and metabolic flux analysis were performed.

### 2.3. Growth profile and spent media analysis

At various points of growth aliquots of medium from each culture were obtained to monitor the evolution of the bacteria growth profile and to analyze changes in medium composition and properties throughout the growth period. Aliquots of 10 mL were centrifuged at 10,000 x g for 15 min to separate the cells. The pelleted cells were solubilized by heat in 1mL of 0.5N NaOH and a
Bradford assay was performed to assess the protein concentration in the cultures at the specific time point of isolation. The supernatant, hereon referred to as the spent medium, was also analyzed. Protein concentration in the spent medium was assessed by Bradford assay and the pH value was noted. To monitor the consumption of glucose in the medium, a phenol-sulfuric acid assay was performed (modified from Saha, 1994). Briefly a sample of spend medium was added to a glass test tube along with 500μL of 4% phenol followed by 2.5 ml 96% sulfuric acid. As the reagents react a color change occurs. The solutions were then transferred to cuvettes and the absorbance was measured at 490nm. Glucose concentration was calculated using a standard curve, plotting the absorbance of samples with known glucose concentration. The spent media were further analyzed by high performance liquid chromatography (HPLC) in order to monitor their metabolite profiles. Samples of 200μL of spent media were treated with 100μL of a 0.5% solution of perchloric acid in mobile phase (20mM HPLC grade KH₂PO₄, pH 2.9 prepared in MilliQ water) and placed on ice for 10 min to precipitate soluble proteins. The precipitate was removed by centrifugation at 10,000 x g for 10 min. Perchloric acid is commonly used for treating samples for HPLC since it provides an accurate measure of organic acids and adenosine nucleotides (technique modified from Samizo, 2001). The supernatant was then collected and filtered directly into a screw top sampling vial using Pasteur pipettes packed with 1mm of cotton. The volume of the
solution in the vials was adjusted by washing the Pasteur pipettes with 700μL of MilliQ water to yield a final volume of 1mL. The sample vials were then loaded into the sampling rack of the HPLC (Waters Alliance 2695 separation module). The automatic injection protocol for the HPLC was programmed with the EMPOWER software. Each injection protocol was initiated by equilibrating the column in the appropriate mobile phase for 120min followed by the injection of 10μL of each sample. The HPLC was calibrated by injecting varying concentrations of standard compounds (5-point calibration). The column used for HPLC was a C18-reverse phase column (3.5μm, 4.6mm x 150mm inside diameter, Symmetry column, Phenomenex ®) with a polar cap operating at a flow rate of 0.7mL/min at 26°C and 1200psi. Metabolites and nucleotides were detected simultaneously using a Waters model 2487 UV-Vis dual wavelength detector operating at 210nm and 254nm. Peaks were quantified using the EMPOWER software and identified by comparing the elution profile to standards and spiking the samples with standard solutions.

2.4. Metabolite profile of the soluble CFE

To monitor the metabolite profile of the cytoplasmic fraction of the control and stressed bacteria, cells were harvested and CFE was obtained as previously described. Protein concentration of the soluble CFE was determined by Bradford assay and a 2mg equivalent was treated with 0.5% perchloric acid in mobile phase. The final volume of the mixture was 200μL.
Like the spent media samples described above, the precipitated proteins were removed by centrifugation and the supernatant was prepared for HPLC. Again, the UV-Vis dual wavelength detector was operating at 210nm and 254nm. The column and mobile phase along with the flow rate, temperature and pressure were the same as for the previously described HPLC procedure.

2.5. Sodium dodecyl sulfate (SDS)- Polyacrylamide gel electrophoresis (PAGE)

Protein in the soluble and membrane CFE of the control and stressed cells were fractionated using SDS-PAGE. SDS-PAGE was performed in a discontinuous buffer system according to a modified method as originally described by Laemmli (Laemmli, 1970). Soluble and membrane proteins were harvested from *P. fluorescens* and quantified by Bradford assay. Fractions were prepared at 1mg/mL in a final volume of 200μL of Laemmli buffer [62.5mM Tris-HCl (pH 6.80), 2% (w/v) SDS, and 2% (v/v) β-mercaptoethanol] and stored at -20°C until needed. Prior to electrophoresis, samples were thawed and boiled for 5 min at 95°C in order to denature all proteins. For SDS-PAGE, 10% isocratic gels were used since they yield the proper separation of the proteins. The concentrations for the separating gels were; 9.2% acrylamide and 0.8% bis-acrylamide, 0.375mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.17% (v/v) TEMED, and 0.86% APS. Once polymerized the separating gel was overlaid with a stacking gel that allowed for proper protein
loading [4% acrylamide and 0.8% bis-acrylamide, 0.1% (w/v) SDS, 0.625mM Tris-HCl (pH 6.8), 0.06% (v/v) TEMED, and 0.03% APS]. Thirty micrograms of protein was then loaded into each well and the electrophoresis was performed at room temperature under denaturing conditions. The electrophoresis buffer (pH 8.3) contained 0.025mM Tris, 0.196mM glycine, and 0.1% (w/v) SDS.

Electrophoresis was performed at 80V throughout the stacking gel. Once the running front entered the separating gel, the voltage was increased to 150V until the bromophenol blue marker exited the bottom of the separating gel. The amperage was not allowed to exceed 50mA. For protein staining, gels were fixed and stained with 10% acetic acid, 50% methanol, and 0.2% Coomassie Brilliant Blue R-250 for 45min at 60°C. Gels were then destained overnight. Kaleidoscope standards and Precision Plus Dual Color molecular mass markers (Bio-Rad) were used to estimate the molecular mass of selected proteins. Table 2 describes the composition of all the buggers used in the SDS-PAGE experiments.

2.6. Monitoring the end-products of oxidative stress

2.6.1. Measurement of oxidized lipids

Thiobarbituric acid (TBA) is known to react with aldehyde equivalents resulting from lipid peroxidation. Therefore the TBA Reactive Species (TBARS) Assay was performed in order to evaluate the content of oxidized
lipids in the membrane of both the control and stressed cultures (technique modified from Aydin, 2005). Briefly, cell membrane fractions were isolated and 2mg of membrane protein equivalent was heated with 15% trichloroacetic acid, 0.375% TBA/0.25N HCl in a final volume of 1mL for 15 min. Following the development of a pinkish color, samples were centrifuged at 10,000 x g for 10 min. The supernatant was isolated and the absorbance was measured at 532nm. Blanks did not contain any membrane fraction and negative reactions were performed in the absence of TBA. The extinction coefficient used was ε = 1.56 x 10^5 M^-1 cm^-1.

Table 2. SDS PAGE buffers:

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide stock (29.2% T and 0.8% C)</td>
<td>29.2 g acrylamide (T) 0.8 g bisacrylamide (C) Stored at 4°C</td>
</tr>
<tr>
<td>4x Tris/SDS pH 6.8</td>
<td>1.5M Tris 0.4% (w/v) SDS pH adjusted to 6.8 with 6N HCl Stored at 4°C</td>
</tr>
<tr>
<td>4x Tris/SDS pH 8.8</td>
<td>1.5M Tris 0.4% (w/v) SDS pH adjusted to 8.8 with 6N HCl Stored at 4°C</td>
</tr>
<tr>
<td>6x Laemmli buffer</td>
<td>7mL of 4x Tris/SDS pH 6.8 3.8g glycerol 10% (w/v) SDS 6% (v/v) β-mercaptoethanol 2mg bromophenol blue</td>
</tr>
<tr>
<td>5x Electrophoresis buffer (1L)</td>
<td>15.1g Tris 72g glycine 5% (w/v) SDS Stored at 4°C</td>
</tr>
<tr>
<td>Destaining solution (1L)</td>
<td>50% methanol 10% acetic acid 40% ddH₂O</td>
</tr>
<tr>
<td>Coomassie R-250 staining solution (1L)</td>
<td>50% methanol 10% acetic acid 40% ddH₂O 0.2% Brilliant Blue R-250</td>
</tr>
</tbody>
</table>
2.6.2. Pyruvate and bromo-pyruvate as antioxidants

In order to assess the antioxidant properties of the ketoacid pyruvate, 1mg of membrane fraction protein equivalents were isolated and centrifuged at 10,000 x g for 10 min in order to separate the membranes from the CSB. The supernatant was discarded and the pellet was resuspended in a solution of 2mM pyruvate [in reaction buffer (50mM Tris-HCl, pH 7.3 with 5mM MgCl₂)] for 30 min. Following a half-hour incubation, the mixture was centrifuged at 10,000 x g to separate the membrane once again from the supernatant. The membrane was then analyzed by the TBARS assay for oxidized lipids (as described above) while the supernatant was collected and treated for HPLC analysis in order to determine the metabolite profile of the reaction mixture following membrane incubation. To ensure the changes observed were not due to pyruvate metabolism, the experiment was repeated with the pyruvate analogue, bromo-pyruvate (Br-pyr). Negative control reactions were performed by incubating the membranes in reaction buffer without pyruvate in order to ensure that the observations were truly an effect of pyruvate incubation.

2.6.3. Measurement of oxidized proteins

The protein carbonyl content was determined in the soluble fraction of the control and H₂O₂-stressed CFE as an indirect assessment of oxidized protein (Frank, 2000). One milligram of protein was mixed with 1mL of 2% (w/v) 2,4-
dinitiophenylhydrazine (DNPH) and allowed to stand for 1h before 200μL of 50% (w/v) trichloroacetic acid was added to precipitate the proteins. DNPH reacts readily with the relevant carbonyl groups of proteins to yield 2,4-dinitrophenylhydrazones. The proteins were then pelleted by centrifugation at 10,000 x g for 10 min. The supernatant was discarded and the protein pellet was washed 3 times in a 10% (w/v) trichloroacetic acid solution. The protein pellet was resuspended and washed 3 times in an ethylacetate:ethanol (1:1 v/v) solution. The resulting pellet was then dissolved in 1mL 6M guanidine-HCl and the absorbance was measured at 370nm. Blanks did not contain any soluble protein and negative reactions were performed in the absence of DNPH. The extinction coefficient for hydrazones was ε = 21.5 nM⁻¹ cm¹.

2.7. Spectrophotometric monitoring of enzyme activity in the CFE

The CFE from *Pseudomonas fluorescens* were isolated as previously described. The protein content of each fraction was measured by Bradford assay. The methods utilized to monitor the various enzymatic activities are described below. For spectrophotometric analyses, the specific activity and standard deviation were calculated for each enzyme. All experiments were performed at least three times and in duplicate unless otherwise indicated. A summary of the techniques used to analyze the enzymes of interested during this work is shown below (Table 3).
2.7.1. Catalase (CAT) activity

The activity of catalase (CAT), EC 1.11.1.6, was measured with the aid of the reagent p-anisidine, and the absorbance at 458nm was monitored. Briefly, 200μg of soluble CFE protein equivalent from the control and H2O2-stressed cells were incubated with 15mM H2O2 in reaction buffer. 10mM p-anisidine was added immediately in a final volume of 1.0mL and the absorbance was measured after 60min. Blanks were prepared similarly with the exception of the addition of H2O2 (technique modified from Igamberdiev, 1995).

2.7.2. Assay for aldehydes and ketoacids

The reagent DNPH was used to assess the levels of aldehydes and ketoacids produced by enzyme reactions. DNPH reacts readily with the majority of biologically relevant aldehydes and ketoacids to yield 2,4-dinitrophenylhydrazones. The reactions were performed initially under acidic conditions, 5mM DNPH in 2N HCl, and the addition of 1N NaOH to deprotonate and colorize the 2,4-dinitrophenylhydrazones. The absorbance was then monitored at 450nm, \( \varepsilon = 16000 \text{ M}^{-1} \text{ cm}^{-1} \).
Table 3. Summary of methods used to monitor the various enzymes of \textit{P. fluorescens} under oxidative stress.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant enzymes</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Spectrophotometric analysis</td>
</tr>
<tr>
<td>TCA cycle enzymes</td>
<td></td>
</tr>
<tr>
<td>NAD(P)-ICDH</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>KGDH</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>MDH</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>FUM</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Oxidative phosphorylation enzymes</td>
<td></td>
</tr>
<tr>
<td>Complex I</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Complex IV</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td></td>
</tr>
<tr>
<td>PDH</td>
<td>Spectrophotometric analysis</td>
</tr>
<tr>
<td></td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Acetate metabolism</td>
<td></td>
</tr>
<tr>
<td>AcK</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>ACC</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Substrate level phosphorylation enzymes</td>
<td></td>
</tr>
<tr>
<td>PK</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>PEPS</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>AK</td>
<td>HPLC</td>
</tr>
<tr>
<td></td>
<td>BN PAGE</td>
</tr>
<tr>
<td>\textit{NADH:NADPH homeostasis}</td>
<td></td>
</tr>
<tr>
<td>NADK</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>ME</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>PC</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td></td>
</tr>
<tr>
<td>GDH</td>
<td>HPLC</td>
</tr>
<tr>
<td></td>
<td>BN PAGE</td>
</tr>
<tr>
<td></td>
<td>Spectrophotometric analysis</td>
</tr>
<tr>
<td>HK</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>G6PDH</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>GPI</td>
<td>BN PAGE</td>
</tr>
<tr>
<td>6PGDH</td>
<td>BN PAGE</td>
</tr>
</tbody>
</table>

The reactions were performed in a final volume of 1mL containing 50mM Tris-HCl, pH 7.3 with 5mM MgCl₂. Prior to stopping the reactions, the samples were
separated into 2 x 0.5mL fractions whereby 0.1mM 2,4-DNPH (5mM in 2N HCl) was added. This stopped the reaction. The samples were allowed to stand at room temperature for 15min. The samples were then diluted with 1mL of ddH₂O and 1mL of 1N NaOH. The absorbance was measured within 10min at 450nm resulting from the presence of the 2,4-dinitrophenylhydrazones. Appropriate controls and blanks were prepared and the respective ketoacids and aldehydes of interest were used as standards.

2.7.3. Pyruvate dehydrogenase (PDH) activity

Pyruvate dehydrogenase (PDH), EC 1.2.4.1, catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, with the concomitant release of NADH from NAD⁺. The activity of this enzyme was monitored in the membrane CFE of control and stressed cells by measuring the consumption of pyruvate with the aid of DNPH. Membrane protein equivalent of 200µg were incubated with 0.2mM pyruvate, 0.1mM CoA, and 0.5mM NAD⁺ in a final volume of 1mL of reaction buffer. The absorbance was measured at 450nm and pyruvate was used as a standard. Blanks and experimental controls were prepared in a similar manner lacking the substrate pyruvate and the membrane protein, respectively.
2.7.4. NAD(P)H-consuming and producing enzymes

Enzyme activity for NADPH producing enzymes were monitored spectrophotometrically by measuring the increase or decrease in absorbance at 340nm caused by the enzymatic reactions taking place in the reaction mixture.

2.7.5. Glucose dehydrogenase (NADP-GDH)

The activity of glucose dehydrogenase (GDH), EC 1.1.1.47, was assessed by monitoring the production of NADPH. Enzyme activity was observed in the membrane CFE of the control and stressed cells. Briefly, 200µg of membrane protein equivalent was incubated with 5mM glucose and 1mM NADP⁺ and the NADPH produced was assessed by measuring the absorbance at 340nm over a period 3 min. Blanks and experimental controls were prepared in a similar manner lacking the substrate glucose and the membrane protein, respectively.

2.8. Blue native polyacrylamide gel electrophoresis (BN-PAGE)

2.8.1. Pouring native gels

Native PAGE was performed with some modifications as described by Schagger (Schagger, 1991). Mini-gels were cast using 1mm spacers with the dimensions of 8 cm x 7 cm using the Bio-Rad MiniProtean™ 2 system. For the proper separation of cytosolic and membrane proteins under native condition, 4-16% linear
gradient separating gels were used. The volume required for the separating gel was 5.8mL, thus 2.9mL 4% acrylamide and 2.9mL of 10% acrylamide solutions were required to yield a working 4-16% linear gradient separating gel. The gradient was prepared employing a gradient former (Bio-Rad) and a peristaltic pump (Fisher). The recipes for each gel solution are provided in Table 4.

Table 4. Gel solution recipe for BN PAGE.

<table>
<thead>
<tr>
<th></th>
<th>16% acrylamide</th>
<th>4% acrylamide</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide mix (48%T and 1.5% C)</td>
<td>937</td>
<td>234</td>
<td>273</td>
</tr>
<tr>
<td>3x BN buffer</td>
<td>967</td>
<td>967</td>
<td>1136</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>223</td>
<td>1699</td>
<td>2000</td>
</tr>
<tr>
<td>75% (v/v) glycerol</td>
<td>773</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td>7.6</td>
<td>9.7</td>
<td>50</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.8</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

(all volumes are in µL)

Following the solidification of the stacking gel the wells were dried with finely cut filter paper. Samples and markers were loaded into their respective wells and then overlaid with blue cathode buffer (buffer recipes are given below). The inner chamber was then filled with the blue cathode buffer and the outer chamber was filled up with anode buffer. Electrophoresis was done at 4°C to maintain the protein in their native state. A voltage of 80V was applied as the protein migrated through the stacking gel. Once the protein had fully travelled the stacking gel, the voltage was increased to 300V and the amperage was set at 15mA. The blue cathode buffer was replaced with a colorless cathode buffer when the running front was halfway through the separating gel. During the electrophoresis the gel was destained by the colorless cathode buffer.
to provide shaper activity bands. The electrophoresis was stopped after the running front moved out of the bottom of the gel.

Table 5: Blue native PAGE buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blue cathode buffer (1L)</strong></td>
<td>8.96g Tricine (50mM) 3.14g BisTris (15mM) 0.2g Coomassie G-250 pH 7 at 4°C</td>
</tr>
<tr>
<td><strong>Colorless cathode buffer (1L)</strong></td>
<td>8.96g Tricine (50mM) 3.14g BisTris (15mM) pH 7 at 4°C</td>
</tr>
<tr>
<td><strong>Anode buffer (1L)</strong></td>
<td>10.45g BisTris (50mM) pH 7 at 4°C</td>
</tr>
<tr>
<td><strong>3x Blue Native (BN) buffer (50mL)</strong></td>
<td>9.84g 6-aminohexanoic acid (1.5M) 1.57g BisTris (150mM) pH 7 at 4°C</td>
</tr>
<tr>
<td><strong>Destaining solution (1L)</strong></td>
<td>50% methanol 10% acetic acid 40% ddH₂O</td>
</tr>
<tr>
<td><strong>Coomassie R-250 staining solution (1L)</strong></td>
<td>50% methanol 10% acetic acid 40% ddH₂O 0.2% Brilliant Blue R-250</td>
</tr>
<tr>
<td><strong>49.5% acrylamide stock (100mL)</strong></td>
<td>48g acrylamide (T) 1.5g bisacrylamide (C)</td>
</tr>
</tbody>
</table>

2.8.2. In-gel activity staining

Membrane proteins were prepared in a BN buffer [500mM 6-aminohexanoic acid, 50mM BisTris (pH 7.0 at 4°C) and 1% β-dodecyl-D-maltoside]. Maltoside is a mild detergent which facilitates the solubilization of membrane-bound enzymes while preserving their activity. Cytosolic proteins were prepared in a similar manner except
maltoside was omitted. The volume of the samples was adjusted to 500μL at the final protein concentration of 4mg/mL. Samples were then stored at -20°C for up to 3 weeks. The membrane samples were incubated on ice for 30 min before they were frozen for storage to allow the maltoside to exert its effect, and the samples were then placed at -20°C for storage. Thirty micrograms of protein were loaded into each lane for electrophoresis. The electrophoresis unit was placed inside a refrigerator at 4°C to allow the use of high voltage and low amperage. Following electrophoresis, the gel slab was removed from the unit and equilibrated in reaction buffer (25mM Tris-HCl, 5mM MgCl₂) for 15 min. The gels were then placed in designated chambers along with reaction buffer (1.5mL of reaction mixture/lane) containing the appropriate substrates, cofactors, coupling enzymes and incubated for the desired time. The majority of in-gel activity assays were performed by visualizing the formation of reduced nicotinamide dinucleotides at the site of enzyme activity. This was achieved by utilizing an electron transfer mechanism which moves electrons from NADH or NADPH to iodonitrotetrazolium chloride (INT) using phenazine methosulfate (PMS) as an electron mediator. Following the acceptance of electrons, INT forms an insoluble pinkish purple formazan which precipitates at the site of immobilized enzyme activity (Figure 27).
Figure 27. The in-gel detection of nicotinamide dinucleotide dependent enzymes by formazan precipitation.

PMS was replaced with 2,6-dichloroindophenol (DCIP) as the electron mediator in the measurement of enzymes which oxidize NADH or NADPH. Once the formazan precipitate band was visible (reactions were performed until an intense band was
noticeable), the gel was placed in destaining solution to stop catalysis. The gels remained in the destaining solution for 24hrs to remove the Coomassie G-250, leaving the pinkish band on a transparent background. Gels were then subsequently documented. Negative reactions were performed without substrate to ensure band specificity. The gels were also stained with Coomassie R-250 in order to ensure equal protein loading. The activity bands were quantified using Scion imaging for Windows (SCION corporation, Frederick, MD). All activity assays were repeated in triplicate and in biological duplicates.

2.8.3. HPLC analysis of enzyme activity bands

In the case of certain activity stains, bands were excised and incubated in a reaction mixture containing their respective substrates. After an appropriate reaction time, the gel was removed from the mixture and the solution was prepared for HPLC analysis. HPLCs were performed in a manner consistent with the method described previously.

2.8.4. In-gel activity staining for TCA cycle enzymes

The in-gel detection of NAD and NADP-dependent isocitrate dehydrogenase (ICDH), NAD-dependent α-ketoglutarate dehydrogenase (KGDH) and malate dehydrogenase (MDH) were visualized using formazan precipitation. As described above, INT is readily reduced by NAD(P)H-generating enzymes (Singh, 2005). To
monitor the activity of these enzymes, the gels were placed in reaction buffer containing 0.1mM NAD(P)\(^+\), 0.5mg/mL INT, 0.3mg/mL PMS and 5mM of the appropriate substrate in order to detect the activity of the desired enzyme. For example for the cytosolic NADP-dependent, and the membrane bound NAD-dependent forms of ICDH, isocitrate was added as a substrate. KGDH of the membrane fraction is NAD-dependent and required the substrate α-KG in order to visualize the enzyme activity in the gel. In addition to the substrate, KGDH also required 0.25mM CoA as a cofactor in the reaction. In the case of MDH, malate was the substrate utilized in the reaction mixture. Fumarase (FUM) activity was probed in the membrane fraction and the enzyme’s activity was detected by coupling the activity to exogenous MDH (Sigma). Briefly, the gel lanes were incubated in 5mM fumarate, 0.5mM NAD, 5 units/mL MDH, 0.5mg/mL INT and 0.3mg/mL PMS and once the activity band became apparent the reaction was stopped with destaining solution.

2.8.5. In-gel activity staining for oxidative phosphorylation enzymes

For the detection of Complex I, the use of an electron mediator was unnecessary seeing as the enzyme can directly transfer electrons to reduce INT, this initiating the formazan precipitation. Proteins from the membrane fraction were fractionated by electrophoresis and the gel lanes were incubated in reaction buffer containing 0.5mM NADH and 0.5mg/mL INT. Unlike any other gel reaction, detection of ETC components requires the added use of 5mM KCN in the reaction buffer to hinder the transfer of electrons to other components of the chain. NADH oxidase
(NADHOX), EC 1.11.1.1, an alternative oxidase to the ETC, will also precipitate formazan in this reaction. Thus two strong band were evident in the gel with complex I appearing much higher in the gel slab within 4 hours of incubation and NADHOX which appears mid-gel within 5 min. In order to detect the activity of Complex IV, the use of INT and PMS are not sufficient since they lack the reductive potential to receive the elections from the enzyme. Therefore, diaminobenzidine (DAB) is used as the electron acceptor which forms a brown precipitate once reduced. The reaction mixture consists of 5mg/mL DAB, 562.5mg/mL sucrose (used to stabilize cytochrome C) and 10mg/mL cytochrome C (Cyt C). Positive control reactions were done with the modification of the reaction buffer containing 5mM KCN.

2.8.6. In-gel activity staining of pyruvate dehydrogenase (PDH)

Pyruvate dehydrogenase (PDH), EC 1.2.4.1, was probed in the membrane fraction of P. fluorescens by incubating the gel lanes in 5mM pyruvate, 0.5mM NAD\textsuperscript{+} and 0.25mM CoA. The addition of 0.5mg/mL of INT and 0.3mg/mL of PMS allowed for the detection of activity via a formazan precipitate forming at the site of catalysis.

2.8.6. In-gel activity staining of acetate metabolizing enzymes

The reversible cytoplasmic enzyme acetate kinase (AcK), EC 2.7.2.1, catalyzes the phosphorylation of acetate using ATP. This enzyme was probed in the reverse reaction where the formation of ATP was used to phosphorylate glucose via
exogenous hexokinase (HK, Sigma). The resulting glucose-6-phosphate (G6P) formed
was utilized by exogenous G6P dehydrogenase (G6PDH, Sigma) which in turn
reduced NADP⁺. This last reduction reaction leads to the formation of the activity
band. The reaction mixture consisted of 5mM acetyl-phosphate, 0.5mM ADP, 5mM
glucose, 5 units/mL of each HK and G6PDH, 0.5mM NADP⁺, 0.5mg/mL INT and
0.3mg/mL PMS in reaction buffer. The in-gel detection of acetyl-CoA carboxylase
(ACC), EC 6.4.1.2, was performed using a method as described in Simonovic, 2004.
This reaction takes advantage of the P₁ liberated as the result of the carboxylation of
acetyl CoA. The reaction entails the complexation of P₁ by ammonium molybdate
[(NH₄)₆ (Mo₇O₂₄)] which forms an insoluble complex at acidic pH at the site of enzyme
activity. In order to stabilize the precipitate, the tertiary amine triethylamine was used
since it is a much stronger base than ammonium ion. Following electrophoresis the
gel was incubated in reaction buffer containing 10mM ATP, 10mM HCO₃⁻, and 1mM
acetyl CoA for 60min. The gel was then rinsed gently with ddH₂O 5 times to remove
any residual P₁. Following the washing step, gels were exposed to a phosphate
precipitation reagent (1.06g of ammonium molybdate in 1.37mL triethylamine and
9.2mL of concentrated HNO₃). Reactions were performed until the precipitate was
visible at the site of enzyme activity. Reaction mixtures lacking HCO₃⁻ served as a
negative control.
2.8.8. In-gel activity staining of substrate level phosphorylation enzymes

The glycolytic pathway enzyme pyruvate kinase (PK), EC 2.7.1.40, has a cytoplasmic subcellular localization in *P. fluorescens*. PK activity was probed by incubating the gel slab in reaction buffer with 5mM PEP, 0.5mM ADP, 0.5mM NADH, 5 units/mL LDH (Sigma), 0.5mg/mL INT and 0.3mg/mL of DCIP. The exogenous LDH metabolized the resulting pyruvate from PK activity thus allowing NADH oxidation to yield a formazan precipitate at the site of catalysis. The detection of phosphoenolpyruvate synthase (PEPS), EC 2.7.9.2, was performed in a similar manner, however ADP was replaced with 0.5mM AMP and 1mM P_. Adenylate kinase (AK), EC 2.7.4.3, catalyzes the transfer of phosphate from one ADP to an other, yielding ATP and AMP. The phosphotransfer enzyme was probed following electrophoresis by placing the gel in reaction buffer containing 5mM ADP, 5mM glucose, 5 units/mL of each HK and G6PDH, 0.5mM NADP^+, 0.5mg/mL INT and 0.3mg/mL PMS. The activity staining of AK required certain finesse in terms of handling the gel seeing as the formazan precipitate would occur on the surface of the gel and could be displaced with harsh manipulation of the slab.

2.8.9. In-gel activity staining of enzymes involved in NADH:NADPH homeostasis

The enzyme NAD^+ kinase (NADK), EC 2.7.1.23, catalyzes the conversion of NAD^+ to NADP^+, thus maintaining the availability of cofactors for NADPH-producing
enzymes. Detection of membrane NADK was possible by incubating the gel slabs in reaction buffer containing 1mM NAD⁺, 5mM G6P, 10 units G6PDH, 3mM ATP, 0.5mg/mL INT and 0.3mg/mL PMS. Pyruvate carboxylase (PC), EC 6.4.1.1, which indirectly influences the levels of NADH and NADPH in a cell, was also probed in the membrane fraction. The reaction buffer contained 5mM pyruvate, 0.5mM NADH, 1mM ATP, 5mM HCO₃⁻, 8 units MDH (Sigma), 0.5mg/mL INT and 0.3mg/mL DCIP. Since NADH oxidase also reacts in this assay, one must pay particular attention to which band is analyzed. The cytoplasmic NADPH-producing malic enzyme (ME), EC 1.1.1.40, was detected by incubating gel slabs in reaction buffer containing 5mM malate, 0.5mM NADP⁺, 0.5mg/mL INT and 0.3mg/mL PMS.

2.8.10. In-gel activity staining of enzymes involved in glucose metabolism

The activity of glucose dehydrogenase (GDH), EC 1.1.1.47, was visualized by in-gel formazan precipitation caused from the reduction of INT by the electron transfer during enzyme catalysis. The membrane protein fraction was ran through the gel and the lanes were incubated in reaction buffer that contained 5mM glucose, 0.5mM NADP⁺, 0.5mg/mL INT and 0.3mg/mL PMS. Hexokinase (HK), EC 2.7.1.1, activity was monitored in the cytoplasmic fraction of P. fluorescens by incubating the fractionated proteins in reaction buffer containing 5mM glucose, 0.5mM ATP, 5 units/mL G6PDH, 0.5mM NADP⁺, 0.5mg/mL INT and 0.3mg/mL PMS. The production of G6P by the enzyme provided the substrate for the exogenous G6PDH which in turn
reduced NADP\(^+\) into NADPH. The electron transfer led to the formation of a formazan precipitate at the site of enzyme catalysis. Glucose-6-phosphate isomerase (GPI), EC 5.3.1.9, was probed in the cytoplasmic fraction. Gels were submersed in reaction buffer containing 5mM fructose-6-phosphate (F6P), 0.5mM NADP\(^+\), 5 units G6PDH, 0.5mg/mL INT and 0.3mg/mL PMS. Glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49, and 6-phosphogluconate dehydrogenase (6PGDH), EC 1.1.1.44, were both probed in the cytoplasmic protein fractions. The reaction buffer for each enzyme contained 5mM G6P and 5mM 6PG respectively, along with 0.5mM NADP\(^+\), 0.5mg/mL INT and 0.3mg/mL PMS. The reaction of G6PDH was left to react for a longer period of time after the appearance of the formazan precipitate band in order to visualize all three active isoforms of the enzyme found in *P. fluorescens*.

**2.9. Metabolic flux analysis**

Phosphotransfer activity through AK and guanylate kinase (GK) was demonstrated by CFE reactions. Briefly, soluble CFE from control and stressed cells were obtained by the method described above and their protein concentration was quantified by Bradford assay. A 200\(\mu\)g protein equivalent aliquot of the soluble CFE was incubated in reaction buffer containing 5mM GDP and 5mM ADP for 20 min. Reactions were stopped by adding 100\(\mu\)L of 0.5% perchloric acid in mobile phase. Samples were centrifuged to remove precipitated protein and the supernatant was prepared for HPLC analysis.
To attain a better understanding of the fate of glucose in \textit{P. fluorescens}, CFE reactions were performed starting from the glycolytic intermediate 6-phosphogluconate (6PG). Soluble CFE from control and stressed cells were obtained by the method described above and their protein concentration was quantified by Bradford assay. A 800\(\mu\)g protein equivalent aliquot of the soluble CFE was incubated in reaction buffer containing 5mM 6PG and 0.1mM NADP\(^+\). Aliquots of 200\(\mu\)L were taken from the reaction after 10 min and 30 min. Adding 100\(\mu\)L of 0.5% perchloric acid in mobile phase to the isolated aliquots stopped the reactions. The samples were then centrifuged to remove precipitated protein and the supernatant was prepared for HPLC analysis.

\textbf{2.10. Statistical analyses}

All experiments were performed at least twice in biological triplicate unless otherwise indicated. The student t test was calculated to determine the significance of the differences in specific activities of various enzymes along with the difference in metabolite levels in control compared to H\(_2\)O\(_2\)-stressed bacteria. If the calculated t value exceeded the tabulated value of 2.78 for \(n=3\) then the means were considered to be significantly different and \(p\) is said to be \(\leq 0.05\).
3. Results

3.1. Growth and physiological parameters in response to H$_2$O$_2$ stress

3.1.1. Growth profile of *P. fluorescens* exposed to H$_2$O$_2$ stress

When *P. fluorescens* bacteria grown on 7.5mM glucose (results refer to these growth conditions unless noted otherwise) were stressed with H$_2$O$_2$ (100µM), the rate of cell growth was slower than the control cells, as the stressed cells reached a stationary phase near 23hrs compared to 21hrs for the control cultures. The initial growth in the control culture presented no significant lag phase whereas in the H$_2$O$_2$ medium exponential cell division did not begin until about 10hrs of incubation. At the stationary phase of growth, both the control and H$_2$O$_2$ – stressed cells reached a similar biomass (approximately 500mg/mL) (Figure 28).

A second stressed culture was inoculated where the dose of H$_2$O$_2$ was increased to 500µM. Cells did not reach a significant biomass under these conditions. For several experiments, the carbon source or the stress was modified. The growth periods for these various conditions are described in table 6. All stressed-cultures experienced a lag phase that led to a longer growth period before reaching the stationary phase.
Figure 28. Growth profile for *P. fluorescens* grown on a defined 7.5mM glucose medium. Biomass was determined by monitoring protein concentration by means of the Bradford assay. Control culture (●), 100μM H₂O₂ (■) and 500μM H₂O₂ (▲). (Note: the addition of a second dose of H₂O₂ after 15hrs did not affect cellular yield.) Cells were harvested at time points denoted by the arrows; control (black) and 100μM H₂O₂ stress (dotted). (n= 3, ± SD.)

Table 6. Time required to reach stationary phase in various growth conditions in *P. fluorescens*.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Growth time prior stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7.5mM glucose)</td>
<td>21hrs</td>
</tr>
<tr>
<td>100μM H₂O₂ (7.5mM glucose)</td>
<td>23hrs</td>
</tr>
<tr>
<td>500μM H₂O₂ (7.5mM glucose)</td>
<td>N/A (limited growth)</td>
</tr>
<tr>
<td>100μM menadione (7.5mM glucose)</td>
<td>29hrs</td>
</tr>
<tr>
<td>Control (15mM xylose)</td>
<td>25hrs</td>
</tr>
<tr>
<td>100μM H₂O₂ (15mM xylose)</td>
<td>30hrs</td>
</tr>
<tr>
<td>Control (20% glycerol)</td>
<td>29hrs</td>
</tr>
<tr>
<td>100μM H₂O₂ (20% glycerol)</td>
<td>37hrs</td>
</tr>
</tbody>
</table>
3.1.2. Spent media analysis: pH, protein concentration and glucose consumption

The spent media in which cells were isolated from at stationary growth were analyzed in order to monitor any difference between the control and H₂O₂-stressed cultures. In stressed cultures, the spent media had a pH of 6.70 while in the spent media from control cultures the pH was 6.81. As for the protein concentration, both the control and stressed media contained small amounts of protein (in the μg/mL range) with the levels higher in the stressed-media (Table 7).

Table 7. pH and soluble protein concentrations in spent fluid from *P. fluorescens* grown on 7.5mM glucose. (n= 3, ± SD, (*) p ≤ 0.05 vs control samples)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100μM H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spent fluid pH at stationary growth phase</td>
<td>6.81 ± 0.02</td>
<td>6.70 ± 0.03</td>
</tr>
<tr>
<td>Spent fluid protein concentration (μg/ml)</td>
<td>10.6 ± 0.1</td>
<td>14.3 ± 0.2 *</td>
</tr>
</tbody>
</table>

The spent media were further analyzed for glucose levels at various time intervals during the growth of the bacteria. Glucose levels were assessed by a phenol-sulfuric acid method (figure 29) and it was observed that glucose consumption was faster in the control cultures. In the 100μM H₂O₂ cultures, glucose consumption did not begin until 8-10hrs after inoculation of the bacteria. The 500μM H₂O₂ cultures showed no glucose consumption. Both the control and 100μM H₂O₂ cultures exhausted the supply of glucose in the media by 20hrs.
**Figure 29.** Utilization of glucose by *P. fluorescens*. Glucose concentrations were determined by means of the phenol-sulfuric acid assay. Control culture (black), 100μM H₂O₂ (dotted) and 500μM H₂O₂ (grey). (n= 3, ± SD.)

HPLC studies were performed on the spent media. Metabolite profiles were obtained. These were characterized by the presence of higher amounts of both pyruvate and acetate in the 100μM H₂O₂ cultures compared to the spent fluid from the control cultures (Figure 30).
Figure 30. Metabolite accumulation in the spent media of *P. fluorescens* cultured in glucose media (210nm). A) Control B) 100μM H₂O₂. (These chromatograms represent 1 biological replicate of 3).

3.2. Metabolomic investigation of the effects of H₂O₂ on *P. fluorescens*.

3.2.1. HPLC analysis of the soluble CFE

Once the spent fluid of the cultures were analyzed, metabolite profiles of the soluble CFE were obtained (Figure 31). HPLC analysis was used to probe for the metabolic remodelling associated with adaptations during oxidative stress. The stress cultures presented an accumulation of pyruvate as the cells reach stationary growth.
This accumulation of pyruvate was significantly accentuated by a double dose of peroxide given at 15hrs (as highlighted in Panel I, figure 31).

Panel I

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control cultures (Ave. peak integration of three biological replicates)</th>
<th>Single dose H2O2-stressed cultures (Ave. peak integration of three biological replicates)</th>
<th>Double dose H2O2-stressed cultures (Ave. peak integration of three biological replicates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>103626 ± 4594.1</td>
<td>122756.7 ± 5850.5</td>
<td>215575 ± 12107.7</td>
</tr>
<tr>
<td>Acetate</td>
<td>31701 ± 1505.6</td>
<td>47370.3 ± 1701.1</td>
<td>72884.7 ± 1598.1</td>
</tr>
</tbody>
</table>
Panel II

A)

![Graph showing relative absorbances for pyruvate, acetate, PEP, and AMP.](image)

B)

![Graph showing relative absorbances for succinate, ATP, ADP, and GDP.](image)

\[(n=3, \pm SD, \text{ (*) } p \leq 0.05 \text{ vs control})\]
Panel III

Figure 31. Metabolite profile of the sCFE obtained by HPLC analysis. **Panel I.** HPLC soluble cellular fraction of *P. fluorescens* grown in glucose. A) Control B) stress with initial 100μM H₂O₂ C) stress with 100μM H₂O₂ and a similar second dose at 15hrs (These chromatograms represent 1 of 3 biological replicates) along with quantified peaks for pyruvate in acetate. **Panel II.** Metabolite profile of the soluble cellular fraction of *P. fluorescens* grown in glucose medium. Control (black), stress 100μM H₂O₂ (dotted), and double-stress 100μM H₂O₂ (second 100μM H₂O₂ dose after 15hrs of growth) (grey) (n= 3, ± SD, (*) p ≤ 0.05 vs control samples). **Panel III.** : Recovery assay. HPLC metabolite profile measured at 210nm in the soluble CFE after 8hr incubation in different media. A) Control cells were incubated in stress medium (100μM H₂O₂) B) Cells from stress medium transferred to control. (These chromatograms represent 1 of 3 biological replicates).

Along with pyruvate accumulation (a 1.2 fold increase for the single dose of H₂O₂ and a 2 fold increase for the double dose of H₂O₂), acetate levels also show a 1.5 fold increase for the single dose of H₂O₂ and a 2 fold increase for the double dosed cells. Other differences in metabolite levels were also observed between the control cells and those exposed to H₂O₂ (figure 31, panel II). For instance, ADP levels were over 3 fold higher in both single- and double-stressed cells, respectively.

Furthermore, the double-stressed cells show a 1.4 fold increase in AMP. The level of
ATP and succinate within the cells are approximately 1.4 fold greater in the control bacteria. Although some metabolites presented differences in concentrations, other metabolites like PEP and GDP did not vary significantly. To further analyse how H₂O₂ – stress altered the metabolite profile of the soluble CFE, *P. fluorescens* cultures (control and 100μM H₂O₂–stress) were grown to confluency, at which point they were isolated from their media and re-inoculated into a different medium. The control cells were inoculated in a 100μM H₂O₂–stress medium and the stressed cells were inoculated in control medium. After 8hrs the cells were isolated and the soluble CFE was analyzed by HPLC (figure 31, panel III). These results revealed that upon exposure to 100μM H₂O₂–stress, the control culture began to accumulate pyruvate (figure 31, panel III, A) and the 100μM H₂O₂–stressed culture incubated in control medium saw a decrease in pyruvate levels (in comparison to the soluble CFE metabolite profile in figure 31, panel I). Hence, H₂O₂ appeared to be the cause of this metabolic reconfiguration. A summary of the observation made during this work is shown in figure 32.
3.2.2. SDS PAGE visualization of protein from soluble and membrane cell-free fractions

The protein profile of both the soluble and membrane fractions isolated from *P. fluorescens* grown on 7.5mM glucose and stressed with 100µM H₂O₂ were compared to that of the control cultures by means of SDS PAGE (figure 33). There was a variation in the nature of the protein bands between the stress and control cultures indicative of changes to the protein levels in the cultures.
**Figure 33.** SDS PAGE of protein from control and 100μM H₂O₂-stressed cell free extracts (difference are indicated by the arrows). These data are representative of 3 independent experiments.

### 3.2.3. The effects of oxidative stress on cellular components

The oxidation of lipids and proteins is a hallmark of exposure to ROS stress. For this reason the extent of lipid peroxidation and protein carbonylation were examined in both the control and the 100μM H₂O₂-stressed cells (Table 8). Three-fold increases in both MDA equivalents and carbonyl residues were observed,
following H$_2$O$_2$ treatment, indicative of significant increases of the oxidized lipids and oxidized proteins content present in the stressed bacteria.

**Table 8.** H$_2$O$_2$ causes the oxidation of cellular components. (n= 3, ± SD, (*) p ≤ 0.05 vs control samples)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100μM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total oxidized lipids</td>
<td>0.76 ± 0.01 μmol of MDA/mg of biomass</td>
<td>2.3 ± 0.2 μmol of MDA/mg of biomass (*)</td>
</tr>
<tr>
<td>Total oxidized Protein</td>
<td>0.021 ± 0.003 nmoles of carbonyl/mg of protein</td>
<td>0.069 ± 0.003 nmoles of carbonyl/mg of protein (*)</td>
</tr>
</tbody>
</table>

3.3. Proteomic variations between H$_2$O$_2$ – stressed and control bacteria

3.3.1. H$_2$O$_2$ induced the activation of the antioxidant enzyme catalase

Catalase, the enzyme that is ubiquitously called upon to detoxify H$_2$O$_2$ was probed by spectrophotometric analysis in the *P. fluorescens* control and H$_2$O$_2$-stressed cultures. A 6 fold increase in the enzyme’s specific activity was observed in the stressed bacteria (Table 9).

**Table 9.** Catalase activity in *P. fluorescens* (n= 3, ± SD, (*) p ≤ 0.05 vs control samples).

<table>
<thead>
<tr>
<th>Specific activity (μmol H$_2$O$_2$ decomposed*mg$^{-1}$*min$^{-1}$)</th>
<th>Control</th>
<th>100μM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.13 ± 0.04</td>
<td>0.82 ± 0.02 (*)</td>
<td></td>
</tr>
</tbody>
</table>
3.3.2. Modulation of the TCA cycle in *P. fluorescens* under ROS insult

Due to the changes in metabolite profiles observed in *P. fluorescens* exposed to H$_2$O$_2$ (figure 31), the activity of selected metabolic enzymes of the bacteria was measured in order to delineate any proteomic adaptations that may arise under ROS toxicity. The TCA cycle enzymes were probed by BN PAGE in-gel activity staining. NAD-dependent ICDH, α-KGDH and FUM activity all showed higher activity in the control culture (figure 34, A, C and E) relative to the stressed-cells. In contrast, NADP-ICDH and MDH activity are both greater in the 100μM H$_2$O$_2$- stressed cultures (figure 34, B and D).

![Figure 34](image)

Figure 34. H$_2$O$_2$ stress and TCA cycle enzymes in *P. fluorescens*: BN PAGE in-gel activity staining. A) NAD-ICDH. B) NADP-ICDH. C) α-KGDH. D) MDH. E) FUM. (Data are representatives of 3 independent experiments).
3.3.3. Effects of exogenous peroxide on the ETC, a source of endogenous ROS

As the TCA cycle is altered during oxidative stress (figure 34), it was important to monitor the effects ROS stress would have on oxidative phosphorylation. BN PAGE in-gel activity staining was used to monitor two enzymes of the ETC, namely complex I and complex IV. The activity of the control enzymes was greater than that of the 100μM H₂O₂-stressed cultures (figure 35). The activity of complex IV was barely detectable in the membrane CFE from H₂O₂-stressed cells.

| Control | 100μM H₂O₂ | Control | 100μM H₂O₂ |

A) ![Image](image1)

-ve controls (≠ NADH)

B) ![Image](image2)

Figure 35. H₂O₂ stress and oxidative phosphorylation: BN PAGE in-gel activity. A) Complex I B) Complex IV. (Data are representatives of 3 independent experiments).

3.3.4. PDH activity is hindered by ROS insult

Pyruvate accumulation was observed in both the spent media and soluble CFE of *P.fluorescens* exposed to 100μM H₂O₂ (figures 30 and 31), therefore it was of interest to investigate the activity of pyruvate metabolizing enzymes. Since the TCA cycle showed significant changes in the stressed cultures, pyruvate dehydrogenase (PDH), the enzyme priming the ketoacid for the Krebs cycle, was probed by BN PAGE...
in-gel activity staining and spectrophotometric analysis in the membrane fraction of *P. fluorescens* (figure 36).

![Figure 36](image)

**Figure 36.** In-gel activity stain of PDH in control, 100μM H₂O₂ and 100μM menadione cultures. (Data are representatives of 3 independent experiments).

**Table 10.** Specific activity of PDH measured spectrophotometrically using the DNPH assay in glucose and xylose cultures exposed to 100μM H₂O₂. (n= 3, ± SD, (*) p ≤ 0.05 vs control samples).

<table>
<thead>
<tr>
<th>Specific activity (μmol<em>mg⁻¹</em>min⁻¹)</th>
<th>Control</th>
<th>100μM H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose cultures</td>
<td>3.1 ± 0.3</td>
<td>1.2 ± 0.7 (*)</td>
</tr>
<tr>
<td>Xylose cultures</td>
<td>2.1 ± 0.1</td>
<td>0.4 ± 0.1 (*)</td>
</tr>
</tbody>
</table>

PDH showed a marked decrease in formazan precipitation during BN PAGE activity staining in the 100μM H₂O₂-stressed culture (figure 36). To validate that the down-regulation of PDH activity was an adaptation to general oxidative stress rather than solely an effect of 100μM H₂O₂ exposure, cells grown on 7.5mM glucose and stressed with 100μM menadione were also probed by BN PAGE in-gel activity staining. Menadione is a generator of superoxide. Once again PDH activity showed a decrease in activity in comparison to the control (figure 36). The specific activity of PDH was
determined using spectrophotometric analysis and the DNPH assay. The 100\(\mu\)M \(\text{H}_2\text{O}_2\)-stressed cells had nearly a 3 fold decrease in activity in contrast to the control bacteria in the glucose cultures (table 10). The assay was repeated in cultures of \(P.\text{fluorescens}\) grown on 15mM xylose in order to elucidate if a different carbon source might impact differently on the regulation of PDH. As demonstrated in the glucose cultures, PDH from xylose cultures had greater activity in the control cells (table 10). Indeed, in this instance PDH was diminished more than 5 fold.

3.3.5. Lipid peroxidation effects are reversed by pyruvate

The accumulation of pyruvate observed in the cytoplasm and the spent media of glucose fed \(P.\text{fluorescens}\) exposed to \(\text{H}_2\text{O}_2\) could in part be explained by inactivation of PDH, yet the reason for simultaneous presence of higher acetate levels (figures 30 and 31) was unaccounted for. To identify the metabolic pathways responsible for the formation of acetate from the ketoacid pyruvate in oxidative environments, membrane fractions from both control and stressed cells were analyzed for oxidized lipids. The control cells, as previously demonstrated contain a lower amount of MDA equivalents than the stressed cells. The membrane from these same cells was incubated in 2mM pyruvate for 30 min followed by the measurement of oxidized lipids via the TBARS assay. Exposure to pyruvate led to a 5 fold decrease in MDA equivalent concentration in the stressed-cells membrane and also a 3 fold decrease in oxidized lipids content in the control membrane (figure 37, panel I). When the reaction mixture originally containing solely 2mM pyruvate was analyzed by HPLC,
both the control and stressed cells mixtures contained acetate, with the stressed cells showing a more prominent decrease in pyruvate content (figure 37, panel II). In order to ensure that the decrease in oxidized lipids content was not due to pyruvate being metabolized by the cell membrane, the same experiment was repeated using the metabolically inactive bromo-pyruvate and similar results were obtained with a marked decrease in oxidized lipids content.

Panel I

![Graph showing oxidized lipids in MDA equivalents (μmol of MDA/mg of biomass)]
Panel II

A)  i) 

![Graph with peak labeled Pyruvate](image)

ii) 

![Graph with peaks labeled Pyruvate and Acetate](image)

B)  i) 

![Graph with peak labeled Pyruvate](image)

ii) 

![Graph with peaks labeled Pyruvate and Acetate](image)
Figure 37. Panel I. Pyruvate as an antioxidant. A) Oxidized lipids levels as measured by the TBARS assay in control (black) and 100μM H₂O₂-stressed (dotted) cells. B) Oxidized lipids in same cells following treatment with 2mM pyruvate for 30 min (n= 3, ± SD, (*) p ≤ 0.5 vs control samples). Panel II. HPLC analysis of the supernatant obtained from the incubation of the membrane fraction with 2mM pyruvate for 30 min. i) time zero ii) after 30 min incubation. A) Control culture. B) 100μM H₂O₂-stressed cells (These chromatograms represent 1 of 3 biological replicates). Panel III. Bromopyruvate as an antioxidant. A) Oxidized lipids levels as measured by the TBARS assay in control (black) and 100μM H₂O₂-stressed (dotted) cells. B) Oxidized lipids in same cells following treatment with 2mM pyruvate for 30 min (n= 1).

3.3.6. Acetate accumulation leads to higher acetate metabolism

Higher acetate levels in the spent media and the soluble CFE of 100μM H₂O₂-stressed cells steered our attention towards acetate metabolism. We first analyzed the enzyme AcK by BN PAGE in-gel activity staining at various time points during the growth of the bacterial cultures.
Figure 38. Panel I. In-gel activity stain for AcK at various intervals of growth. A) control 15 hrs, B) control 20hrs, C) control 24hrs, D) stress 15hrs, E) stress 20hrs. F) control 20hrs negative control (without acetyl-phosphate), G) stress 20hrs negative control (without acetyl-phosphate) (Data are representatives of 3 independent experiments). Panel II. Densitometric analysis of panel I activity bands using Scion imaging. Panel III. HPLC monitoring of acetate levels in the soluble CFE at various time points of growth (n= 3, ± SD).
**Figure 38 (continued). Panel IV.** Activity bands were excised from the 20hr time point lanes of control and stressed cells followed by incubation in a solution of 5mM acetyl-phosphate and ADP for 30 minutes. The reaction mixtures were analyzed by HPLC in order to confirm enzyme activity. A) time zero B) control C) 100μM H_{2}O_{2} stress (n=1)

At the 15hr time point of growth, the stressed cells presented a more prominent formazan precipitate band, indicative of higher AcK activity in comparison to the control (figure 38, panel I, D and A). This trend continued at the 20hrs time point, which can be seen in the densitometric quantification of the bands where the stress culture exhibited a 2 fold higher pixel density compared to the control (figure 38, panel III, E and B). In the soluble CFE of the control cultures, acetate levels increased over time in a fashion that mirrored AcK activity. In the stressed culture sample however, acetate levels were at the highest levels at 15 hrs and they decreased with the enzyme activity at the 20 hrs time point (figure 38, panel II). Negative controls were performed using protein from the 20 hrs time points of growth. The reaction mixture lacked acetyl-phosphate, indicating that the band seen in figure 38, panel I, require the metabolism of acetyl-phosphate. In addition, activity bands from the 20 hrs time points were precision excised following the appearance of the formazan precipitate and incubated in a reaction mixture containing 5mM acetyl-phosphate and 0.5mM ADP for 30 min. Following incubation the mixture was analyzed by HPLC to confirm the identity of the enzyme. Both the control and stress activity bands were able to use the substrates in order to produce ATP (figure 38, panel IV).
In addition to AcK activity being up-regulated in the stressed cells during peroxide insult, the enzyme ACC also increased in activity with a 2 fold greater activity band, as evidenced by densitometric analyses, in comparison to the control (figure 39).

![Figure 39. A) In-gel activity staining for ACC (Data are representatives of 3 independent experiments) B) Formazan precipitate pixel density for the ACC in-gel activity stain, obtained using Scion imaging.](image)

3.3.7. Substrate level phosphorylation increases during oxidative stress

Oxidative stress led to a decline in the availability of ATP (as observed in figure 31, panel II) due to the lack of activity of the ETC complexes (figure 35). With oxidative phosphorylation being inhibited, other metabolic pathways leading to ATP synthesis must be activated if the organism is to maintain its growth profile. The involvement of ADP-dependent enzyme PK, and its AMP/Pi-dependent counterpart PEPS, in ATP
synthesis was assessed using BN PAGE in-gel activity staining (figure 40). PK activity saw no dramatic change in activity between the control and the stressed cultures (figure 40, A), however PEPS saw over a 10 fold increase in activity (according to pixel density) in the cells exposed to H$_2$O$_2$ (figure 40, B-C). This increase in activity was reversed by re-inoculating the bacteria in opposing medium for 8 hrs (figure 40, D). The importance of this substrate level phosphorylation system was further investigated in *P. fluorescens* cultures grown on xylose and glycerol. As in the glucose cultures, the in-gel activity staining for PEPS led to a great formazan precipitate in the H$_2$O$_2$-stressed lanes of the gel for the cultures fed with these alternate carbon sources (figure 40, E-F).

Figure 40. Substrate level phosphorylation under oxidative stress. A) In-gel activity stain for PK, B) in-gel activity stain for PEPS in glucose cultures, C) pixel density analysis of activity bands of PEPS in glucose cultures, D) Induction of PEPS activity by re-inoculation of control cultures in 100μM H$_2$O$_2$ media for 8 hrs and suppression of PEPS activity in stressed-cells re-inoculated in control media.
**Figure 40 (continued).** E) PEPS in-gel activity stain in xylose cultures, F) in-gel activity stain for PEPS in glycerol cultures. (Data are representatives of 3 independent experiments).

In order to confirm the identity of the enzyme probed by BN PAGE in-gel activity staining seen in figure 40B, the activity bands from the control and stressed CFE were excised and incubated in a reaction mixture containing 5mM PEP, 0.5mM AMP and 1mM P_i. The enzyme was confirmed by the production of both ATP and pyruvate from these substrates along with the consumption of PEP. The peaks were more pronounced in the stressed cultures, with a 10 fold increase in pyruvate production, a 4 fold increase in ATP production and a 1.3 increase in PEP utilisation respectively compared to the controls (figure 41).

**Figure 41.** Substrate level phosphorylation activity through PEPS. Activity bands were precision excised and incubated in reaction mixtures containing 5mM PEP, 0.5mM AMP and 1mM P_i for 60 min and analyzed by HPLC. A) PEP consumption.
Figure 41 (continued) B) Pyruvate and ATP production. Control (black), 100μM H$_2$O$_2$-stressed (dotted) (n= 3, ± SD, (*) p ≤ 0.05 vs control samples).

3.3.8. H$_2$O$_2$-toxicity promotes phosphotransfer systems in *P.fluorescens*

An increase in the activity of AMP dependent enzyme such as PEPS along with the need to store the high energy contained in ATP, led us to examine whether the enzyme AK was induced in the H$_2$O$_2$-stressed bacteria (figure 42).

Panel I

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
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-ve controls (- ADP)
Figure 42. A ROS inducible phosphotransfer system through AK. Panel I. A) In-gel activity stain for AK in control 15hrs cultures, B) In-gel activity stain for AK in control 20hrs cultures, C) In-gel activity stain for AK in control 24hrs cultures, D) In-gel activity stain for AK in stress 15hrs cultures, E) In-gel activity stain for AK in stress 20hrs cultures, F) In-gel activity stain for AK in stress 24hrs cultures, G) Induction of AK activity by re-inoculation of control cultures in 100μM H₂O₂ media for 8hrs, H) suppression of AK activity in stressed-cells re-inoculated in control media for 8hrs, I) negative control (without ADP) in control 20hr culture, J) negative control (without ADP) in stress 20hr culture (Data are representatives of 3 independent experiments).
Figure 42 (continued). Panel II. The activity bands were precision excised and incubated with 5mM ATP and 5mM AMP for 60 min. The reaction mixtures were analyzed by HPLC. i) time zero ii) 60 min incubation. A) Control culture. B) 100μM H₂O₂-stressed cells. (n= 1)

Over the course of the culture growth, AK activity is greater in the bacteria subjected to oxidative stress in comparison to the control at every time point analyzed, with the highest activity being observed at 20hrs (figure 42, panel I, A-F). When the control cells were re-inoculated in stress medium, AK activity was induced and the reverse trend was observed when stressed cells were re-inoculated in control medium (figure 42, panel I, G-E). Negative controls were performed using protein from the 20 hrs time points of growth. The reaction mixture lacked ADP, indicating that the enzymes visualized in figure 42, panel I, require this metabolite in order to form a formazan precipitate. Further confirmation of the enzyme was achieved by excising the activity bands from the 20hrs time and incubating them in a reaction mixture containing 5mM ATP and 5mM AMP for 60 min. The reaction mixture was then analyzed by HPLC (figure 42, panel II).

The phosphotransfer systems of P.fluorescens were monitored using HPLC analysis of reaction mixtures containing soluble CFE, GDP and ADP. After only 20 min incubation, the enzymes of both the control and the stressed cells were able to produce a variety of substrates from the two diphosphonucleotides such as ATP, GTP and AMP. There were, however, no significant differences between the amounts of products formed between the control and the stress CFE (figure 43).
3.3.9. NADH homeostasis is modulated by enzymes evoked under ROS stress

NADH production is not pivotal for the growth of *P. fluorescens* under conditions that do not favour oxidative phosphorylation, for example ROS insult. However the production of NADPH, the reducing power of many antioxidant systems, is crucial. For this reason, enzymes involved in NADH/NADPH homeostasis were probed using BN PAGE in gel activity staining techniques.
Figure 43. Phosphotransfer activity through adenylate kinase and guanylate kinase is demonstrated by HPLC analysis. 0.200mg of soluble CFE was incubate with 5mM GDP and 5mM ADP for 20 minutes. HPLC analysis was used to monitor the evolution of the metabolite profile during the reaction. i) Time zero. ii) After 20 minute incubation A) Control cultures  B) 100\(\mu\)M \(\text{H}_2\text{O}_2\)-stressed cultures. (n= 3).
Figure 44. BN PAGE in-gel activity stains for enzymes involved NADH and NADPH homeostasis. A) MDH. B) NADK C) PC. D) NADH oxidase. E) ME (Data are representatives of 3 independent experiments).

The activity of MDH, NADK, PC, NADH oxidase and ME were all visualized by formazan precipitation on the gel slabs. These enzymes showed greater precipitate formation in the stress protein lanes of the gel, indicative of a higher activity in comparison to the control (figure 44).

3.3.10. Glucose metabolism is geared towards NADPH production when *P.fluorescens* is exposed to oxidative stress

In-gel activity staining techniques were utilized to investigate the differences in glucose metabolizing pathways used by the control and stressed cells. The enzymes
HK and GPI both underwent a decrease in activity in cultures exposed to oxidative stress, whether it be H$_2$O$_2$ or menadione (figure 45, A-B). GPI was also observed to have a decreased activity in comparison to the control in cells grown on 15mM xylose and 100μM H$_2$O$_2$ (figure 45, C).

![Image A)](image)

![Image B)](image)

![Image C)](image)

**Figure 45.** In-gel activity staining of A) HK in glucose control, 100μM H$_2$O$_2$, 100μM menadione cultures, B) GPI in glucose control, 100μM H$_2$O$_2$, 100μM menadione cultures, C) GPI in xylose control and 100μM H$_2$O$_2$ cultures (Data are representatives of 3 independent experiments).

Since HK activity was decreased under oxidative stress, the membrane bound, NADPH producing GDH was probed. This enzyme was first visualized by BN PAGE in-gel activity staining which resulted in a greater activity in the 100μM H$_2$O$_2$ and
100μM menadione cultures in contrast to the control cultures (figure 46, panel I). The identity of the activity band was confirmed by incubating the excised bands from the control and 100μM H₂O₂ cultures, in a reaction mixture containing 5mM glucose and 0.5mM NADP⁺. The production of gluconate, the product of GDH, was achieved by both the control and stress activity bands (figure 46, panel II). The control cells were re-inoculated in stress media, which lead to the induction of GDH activity. The trend was reversed when stressed cells were re-inoculated in control media (figure 46, panel III). When the specific activity of GDH was monitored spectrophotometrically through the production of NADPH, the 100μM H₂O₂ culture had a 2 fold higher activity than the control (Table 11).

As NADPH production was being upregulated through the activation of GDH, other downstream NADPH producing enzymes were investigated. Notably, G6PDH and 6PGDH showed great activities in the peroxide-stressed cells. In the case of G6PDH, the stressed cultures presented three different isozymes, visualized by BN PAGE in-gel activity staining (figure 47, A). The band quantification of G6PDH seen next to the gel in figure 47A, confirmed approximately a 2 fold increase in pixel density in the lowest band from the stressed cultures. Similarly, 6PGDH, the initiating enzyme for the pentose phosphate pathway, was more active in the stressed cell sample than the control (figure 47B).
Panel I

Control 100μM H₂O₂ 100μM Menadione

Panel II

Figure 46. GDH activity. Panel I. In-gel activity staining of A) HK in glucose control, 100μM H₂O₂, 100μM menadione cultures. (Data are representatives of 3 independent experiments) Panel II. NADP-dependent GDH activity was confirmed by excising the activity bands from the BN PAGE gel and incubating it with 5mM glucose and 0.5mM NADP for 30 minutes. The reactions mixture was analyzed by HPLC. A) Control culture. B) 100μM H₂O₂-stressed cells. (n= 3)
Figure 46 (continued). Panel III. In-gel activity staining for regulation of GDH activity. GDH activity provoke by re-inoculation of control cultures in 100µM H₂O₂ media for 8hrs and suppressed in stressed-cells by re-inoculating in control media for 8hrs. (Data is representative of 3 independent experiments).

Table 11. GDH specific activity monitored by NADPH production (n= 3, ± SD, (*) p ≤ 0.05 vs control samples)

<table>
<thead>
<tr>
<th>Specific activity (nmol NADPH produced<em>mg⁻¹</em>min⁻¹)</th>
<th>Control</th>
<th>100µM H₂O₂</th>
</tr>
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<tbody>
<tr>
<td>1.34 ± 0.01</td>
<td>2.82 ± 0.04 *</td>
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Figure 47. In-gel activity staining for A) G6PDH along with Scion imaging densitometry data, B) 6PGDH. (Data are representatives of 3 independent experiments)
Increases in activity of G6PDH and 6PGDH led to the investigation of the involvement of the pentose phosphate pathway in glucose metabolism as a consequence of H$_2$O$_2$ stress. To delineate whether the pentose phosphate pathway was activated soluble CFE from control and stress cultures were incubated in a reaction buffer containing 6-phosphogluconate, and aliquots of the reaction at various time points were analyzed by HPLC (figure 48). As soluble CFE from control and stressed cells were incubated with 6PG, the control cultures made a significant greater amount of pyruvate than the stressed cultures did after 30 min of incubation (figure 48, panel I, A, ii-iii). The stressed cells however showed production of both fructose-6-phosphate (figure 48, panel I, B, ii) and G3P (figure 48, panel I, B, ii-iii). Hence, it appears that the pentose phosphate pathway was enhanced in the H$_2$O$_2$-stressed cells.
Figure 48. Panel I. Metabolism of 6PG by soluble CFE in *P. fluorescens*. 0.800 mg of soluble CFE was incubated with 5mM 6PG. Aliquots were collected at i) 0, ii) 10 and iii) 30 minute intervals and analyzed by HPLC. A) Control culture. B) 100μM H$_2$O$_2$-stressed cells. (n= 3).
4. Discussion

A body of literature regarding the survival of living organisms in an oxidative environment exists. The subject of oxidative stress has been explored for decades now and still new insights on the mechanisms of adaptation are being unravelled. Recent discoveries include the involvement of ROS in several diseases as well as cell signalling (Brookes, 2006). It is obvious that a cell must exert incredible control over the levels of ROS in its environment in order to cope with the burden these toxic moieties can impose. The current study focuses on the adaptations evoked in the gram-negative bacteria *P. fluorescens* when faced with an insult of H$_2$O$_2$. Although this work confirms some of the classical concepts related to H$_2$O$_2$ toxicity and detoxification, it reveals the role of pyruvate as an antioxidant and provides seminal findings on ATP homeostasis in H$_2$O$_2$-challenged *P. fluorescens*, a concept that hitherto has never been explored.

4.1. The targets of H$_2$O$_2$ stress

In the presence of H$_2$O$_2$, *P. fluorescens* experienced a lag phase in their growth in comparison to the control cells. This delay in growth, which could represent the time the microbe needed to adapt to the stressing agent, was associated with the initial limited glucose consumption in the H$_2$O$_2$ – stressed cultures (figure 28 and 29). When the dose of H$_2$O$_2$ was increased from 100µM to 500µM, cells were unable to reach a substantial biomass. This indicates that the 500µM dose is beyond the tolerance
threshold of this organism. In addition to differences in growth profile, the control and stressed cells differed in oxidized lipids and protein content, with the levels being higher in the H$_2$O$_2$ – exposed cells (Table 8). Oxidation of cellular components is a key indicator of ROS toxicity (Catalá, 2009; Lyon, 2009). To deal with the onslaught of H$_2$O$_2$, stressed-cells evoke the ubiquitous antioxidant enzyme catalase. The apparent specific activity of catalase was shown to be 8 fold higher in the stressed bacteria compared to the control (Table 9), a trend common in microbes under oxidative stress (Cuéllar-Cruz, 2008; Hébrard, 2009; Ziegelhoffer, 2009). Together, these data show that the peroxide stress is indeed being felt by P.fluorescens and that there are efforts by the cells to adapt, ultimately enabling the stress culture to grow to a similar biomass as the control.

Although catalase has been shown to be a very effective H$_2$O$_2$ detoxifier (Kirkman, 2006), ROS still manage to penetrate the cellular defenses and cause damage. It has been established that [Fe-S] clusters of proteins are primary targets for oxygen radicals (Djaman, 2004). Certain steps involved in the central metabolic pathway, the TCA cycle, require electron transfer through [Fe-S] clusters. For this reason, enzymes of this pathway were investigated. As expected from previous studies (Fedotcheva, 2006; Mailloux, 2011; Singh, 2009b), modulation of the TCA cycle occurs under oxidative stress conditions (figure 34). There is a shift from NADH production to enhanced NADPH synthesis via the down-regulation of NAD-ICDH and the up-regulation of cytoplasmic NADP-ICDH (Lemire, 2010b; Singh 2008).
Furthermore, KGDH and FUM activities were both reduced in the stressed cells in comparison to the control samples. In addition to TCA cycle modification, the ETC, the site of oxidative phosphorylation and location of a concentration of [Fe-S] clusters, was also monitored. The ETC has been shown to be a contributor of endogenous sources of ROS by inefficient electron transport. Therefore, the ETC, along with the production of its substrate, NADH, have been shown to be shut down when a cell is faced with additional ROS stress (Mailloux, 2011; Singh, 2008). The decrease activity in the oxidative phosphorylation machinery was observed in the 100μM H₂O₂-stressed cultures (figure 35). Hence, this study corroborates previously reported findings on the influence of ROS on the TCA cycle and oxidative phosphorylation. Thus it is clear that H₂O₂ stress mimics other cellular challenges that generate ROS (figure 49). For instance, aluminium (Al), gallium (Ga), zinc (Zn) and menadione, that are known to create an intracellular oxidative environment by favouring the formation of such ROS as H₂O₂, O₂•⁻ and •OH, force major metabolite shifts in numerous organisms (Chénier, 2008; Lemire, 2008; Mailloux, 2008). The modulation of central metabolic pathways such as the TCA cycle led to the hypothesis of further modifications to metabolism being evoked by the stressed cells in order to counter the threat of ROS toxicity. In addition, due to a down-regulation of the ATP-producing ETC, our attention was focused on alternate energy harvesting systems of *P. fluorescens* that would allow the stressed cells to meet their energy needs.
**Figure 49.** The influence of H$_2$O$_2$ insult on *P. fluorescens*.

### 4.2. ROS scavenging by pyruvate leads to an enhanced acetate metabolism

Organisms thriving in aerobic environments are equipped with an arsenal of ROS-scavenging molecules, with GSH and ascorbate being some of the most studied (Shao, 2008; Ros, 2000). A recent flood of evidence has shown that under oxidative stress, metabolic intermediates known as α-ketoacids are accumulated for their ROS-detoxifying properties. The accumulation of these metabolites, like α-KG, oxaloacetate and pyruvate can be achieved through modulation of the enzymes responsible for their metabolism. This type of oxidative stress response has been shown in numerous organism including bacteria such as *P. fluorescens*, the amitochondriate diplomads *Giardia intestinalis* and *Hexamita inflate* and in the mitochondria of mammalian cell lines such as human and rat hepatocytes (Mailloux, 2011; Brookes, 2006; Fedotcheva, 2006; Biagini, 2001). The antioxidant capacity of α-ketoacids comes from their ability to react non-enzymatically with H$_2$O$_2$, which leads to the decarboxylation of the respective carboxylic acid and thus nullifying the reactivity of the oxygen derivative.
Figure 50. Non-enzymatic decarboxylation of metabolically relevant ketoacids reacting with $\text{H}_2\text{O}_2$ ($\text{O}_2^-$ and $\cdot\text{OH}$ and reactive nitrogen species (RNS) react in an analogous manner).

In the $\text{H}_2\text{O}_2$-stressed *P. fluorescens*, pyruvate levels increased in the soluble cell-free extract and the spent medium in comparison to the control samples (figure 30 and 31). Returning the cells from the stressed cultures to the control media (or vice-versa) reversed the change in pyruvate levels in these cells, therefore indicating that the oxidative stress was the determining factor for pyruvate accumulation (figure 31, panel III). The build-up of the $\alpha$-ketoacids is advantageous as it is a proven ROS-scavenging agent in various scenarios including the prevention of cataract formation in the eye, protection of the myocardium during post-ischemia ROS insult, and allows for cell survival in single-cell microbes in oxidative environments, to name a few.
(Hedge, 2007; Hedge 2005; Mailloux, 2011; Mallet, 2002). The accumulation of pyruvate is facilitated by the inhibition of PDH by oxidative agents, a process that occurs in a similar way as KGDH inhibition. PDH activity is hindered by both H₂O₂ and O₂⁻ (menadione) in the glucose cultures and by H₂O₂ in xylose cultures, indicating the importance of this metabolic adaptation (figure 36, table 10). What makes PDH, along with other ketoacid dehydrogenases, so susceptible to ROS stress is the covalently bound lipoic acid cofactor. It has been shown that oxidation products in the lipid membranes can react with membrane bound proteins. The aldehydic product of lipid peroxidation, 4-hydroxy-2-nonenal (HNE), reacts with the lipoic acid of PDH, leading to the disruption of enzyme activity (figure 51) (Humphries, 1998). The addition of lipoic acid has been shown to partially reverse this trend (Singh, 2009a).

To further explore the antioxidant properties of pyruvate, its reactivity with oxidized lipids was used to test the ketoacid’s ability to decrease ROS toxicity. When cell membranes were incubated in 2mM pyruvate for 30 min, the levels of oxidized lipids were reduced significantly (figure 37, panel I). The reaction mixture was analyzed by HPLC, which showed a consumption of pyruvate and the accumulation of the non-enzymatic decarboxylation by-product acetate (figure 37, panel II). These data support the concept of pyruvate having a ROS-scavenging ability and would explain why the stressed cell would accumulate the metabolite. In order to show that the decrease in oxidized lipids was not due to pyruvate metabolism Br-pyruvate, which cannot be metabolized, was used. Again, in the presence of this derivative of
pyruvate, the oxidized lipids levels were decreased with the concomitant formation of bromoacetate (figure 37, panel III).

![Diagram of chemical reaction]

**Figure 51.** Reaction of lipoic acid with HNE followed by reductive stabilization (Humphries, 1998).

As acetate formation is the result of the reaction of pyruvate with ROS, it was
no surprise to observe an increase in acetate levels in the cytoplasm and the spent medium of the H₂O₂-stressed cells (figure 30 and 31). The accumulation of acetate during oxidative stress has been observed by other groups. Taya et al. studied the effect of ROS caused by the photoexcitation of titanium dioxide in SOD-deficient E. coli (Taya, 2008). The stressed cells grown on glucose showed accelerated acetate accumulation in the medium compared to the control cultures. It was, however, concluded that oxidative stressed E.coli was more prone to acetate metabolism. The current study not only demonstrates this possibility, but expands the notion that acetate is generated as a consequence of pyruvate being utilized as a ROS scavenger. Increased acetate metabolism occurs as well in P. fluorescens stressed with peroxide where the cells presented a higher activity of AcK, notably at the 15hrs of growth that coincides with the introduction of the second dose of H₂O₂. The control cells eventually acquired a need for acetate metabolism upon reaching stationary growth and exhausting their glucose supply (figure 38). Acetate kinase activity was complemented with another enzyme, ACC, which is indirectly associated with acetate metabolism. ACC also underwent an increase in activity during oxidative stress conditions. This enzyme, which generates acetyl-CoA for liponeogenesis, could be experiencing a spike in activity to allow for the regeneration of the cell membrane components damaged by ROS (i.e. the oxidized lipids). Ultimately the exposure to ROS leads to an increase of pyruvate accumulation and the subsequent need for acetate metabolizing enzymes (figure 52).
Figure 52. Pyruvate is used by *P. fluorescens* to detoxify ROS and subsequent acetate formation is dealt with an increase in acetate metabolism.

4.3. Substrate level phosphorylation and phosphotransfer systems in ATP homeostasis

As oxidative phosphorylation showed a decrease in activity in the H$_2$O$_2$-stressed cells, it became critical to understand how this microbe was fulfilling its need in ATP, especially since there appeared to be no marked change in cellular yield in the control and H$_2$O$_2$-stressed cells. As photosynthetic pathways were out of the question and oxidative phosphorylation was hindered, substrate level phosphorylation (SLP) remained the only other option. Under various stress conditions microbes evoke the ATP-producing power of direct substrate phosphorylation by high energy phosphate metabolites (Singh, 2009b). This adaptation allows for the maintenance of ATP homeostasis in order to meet energy demands. The microbe *Trypanosoma brucei*, which is responsible for human sleeping sickness, does invoke a type of SLP in order to meet its energy needs. This organism uses an acetate succinate CoA transferase/succinyl CoA synthetase cycle to generate ATP. In this cycle, the transfer
of CoA from acetyl-CoA to succinate allows the storage of energy in the thioester bond of succinyl-CoA. The succinyl-CoA is subsequently utilized to generate ATP by succinyl CoA synthetase (SCS) (Bochud-Allemann, 2002). A modification of this SLP system has been shown in *P. fluorescens*. During oxidative stress evoked by aluminum exposure, the soil microbe modulates its TCA cycle leading to the formation of glyoxylate through the enzyme isocitrate lyase (ICL), a process known as the glyoxylate shunt. Acetylating-glyoxylate dehydrogenase (AGODH) utilizes the glyoxylate and forms NADPH, a crucial factor to ROS detoxification. Oxalate CoA-transferase (OCT) yields oxalate and succinyl-CoA. The SCS system then efficiently produces ATP. The production of oxalate is also advantageous under these conditions, as it chelates aluminum for its sequestration (figure 53) (Singh, 2009b). Eukaryotes, on the other hand, have two isoforms of SCS. One isoform utilizes ADP as the substrate for the production of ATP, while the second forms GTP from GDP (Singh, 2009b).

Glycolysis is another metabolic network that can produce ATP via substrate-level phosphorylation and numerous cellular systems rely on glycolysis to meet their energy requirements. In this study SLP was investigated at the glycolysis level since glucose was used as a carbon source. PK activity did not vary significantly between the control and the H₂O₂-stressed cultures. However the activity of PEPS, the homologue to the plant pyruvate water dikinase, was far greater in the stress cells (figure 40).
Figure 53. Aluminum toxicity in *P. fluorescens* leads to enhanced SLP via the glyoxylate shunt and the succinyl-CoA (SCS)/oxalyl-CoA transferase (OCT) system (Singh, 2009b).

This increase was observed with all 3 carbon sources used during this study (glucose, xylose and glycerol) and reversed by stressed cells were returned to control media (and vice versa). This enzyme uses PEP, AMP and inorganic phosphate as substrates to produce ATP and pyruvate. The implications of this metabolic adjustment are quite
significant. Using AMP rather than ADP (for PK) is indicative of an effort by the ROS-stressed cells to be frugal with their ATP supply. In other words, they are bypassing the AMP → ADP step that requires an ATP as a phosho-donor, and directly making ATP from AMP (figure 54).

![Diagram of ATP generating network](image)

**Figure 54.** ATP generating network in *P. fluorescens* exposed to H₂O₂.

The production of ATP from PEPS is an important adaptation under oxidative conditions that causes a halt of the ATP-generating respiratory chain. However, for this system to be effective, a phosphotransfer system must be in place to regenerate the nucleotide substrates (i.e. AMP). Phosphotransfer systems are used in the ETC of all organisms. In eukaryotes, phosphotransfer wires are utilized to allow the energetic support by the mitochondria for the nucleus and allow for cell division by the formation of other nucleotides that make up the genetic structure of a cell. Mitochondria clustered around the nucleus generate the majority of ATP required for nuclear processes. Export of ATP from the mitochondrial intracristal space is facilitated by
reactions catalyzed by mitochondrial isoforms of AK, creatine kinase (CK) and NDPK (Dzeja, 2003; Saks, 2006). Once out of the mitochondria the high-energy phosphates are transferred through a phosphor-wire to ATP consumption sites at the nuclear envelope and inside the nucleus by cytosolic and nuclear isoforms of AK, CK and NDPK. Interaction and complementation between these systems ensure proper nucleotide ratios at and across the nuclear envelope, sustaining the high energy of ATP and GTP hydrolysis (figure 55) (Dzeja, 2003).

**Figure 55.** Energy support relays for nucleocytoplasmic communication. ATP synthase produces ATP and the high-energy phosphate is transferred from one intermediate to another by AK, (also possible with CK and NDPK where creatine and creatine phosphate or NDP and NTP are the respective intermediates).
Figure 55 (continued). This maintains the substrates needed for ATP synthesis in the mitochondria and the intermediates are navigated to the nucleus to support the energy and nucleotide demand in the nucleoplasm (Dzeja, 2003).

AK activity was found to be higher in the H$_2$O$_2$-stressed cells at various time points of growth (figure 42). This trend was reversed when stressed cells were returned to control media (and vice versa). This last observation is indicative of the influence of ROS toxicity on the activity of the enzyme. This enzyme works in concert with NDPK to maintain a pool of nucleotides necessary for cellular survival (figure 43). As the high-energy phosphate is being transferred to various storage molecules or used up by metabolic processes, AK allows for the regeneration of AMP for PEPS and the formation of another ATP, from two ADP molecules (figure 56).

![Diagram](image)

**Figure 56.** A novel phosphotransfer network fueled by PEPS, AK and NDPK in *P. fluorescens.*
Adenylate kinase has been proposed to be a virulence factor in *P. aeruginosa* isolated from cystic fibrosis patients. To survive in the hostile environment of the human body, this pathogen secretes AK, in order to disrupt the external ATP supply required by macrophages. This change in nucleotide availability leads to enhanced cell death through P2Z receptor activation which is responsible for the formation of membrane pores in the macrophages (Chakrabarty, 2001). Considering the fact that oxidative stress increases AK activity in *P. fluorescens*, it would be of interest to determine if the oxidative burst used by the macrophages to destroy pathogens induces the secretion of AK in *P. aeruginosa*. The use of ROS by the macrophages might consequently be leading to their demise by stimulating the pathogens own defense arsenal. Hence, ROS may be the cause of AK up-regulation in *P. aeruginosa*. Nonetheless, in conditions where oxidative phosphorylation is incapable of supplying the energy demands of *P. fluorescens*, this microbe invokes a combined SLP and phosphotransfer system to produce ATP.

4.4. **Glucose metabolism plays a major role in NADPH and pyruvate production**

As the focus of this work was to delineate the metabolic adaptation strategies enabling *P. fluorescens* grown on glucose to survive H$_2$O$_2$ toxicity, it was important to investigate the metabolism of this carbon source. Furthermore, it was pivotal to understand if metabolic reconfigurations are contributing to the production of NADPH and pyruvate, two ingredients essential for survival under H$_2$O$_2$ challenge. It has been
previously demonstrated that the need for NADH decreases during oxidative stress and the demand for the antioxidant NADPH is increasingly significant (Mailloux, 2011; Singh, 2008). NADPH is the reducing equivalent utilized to regenerate many antioxidant systems and these ROS detoxifiers are exhausting the NADPH resources during oxidative stress. For this reason, cells favor metabolic pathways leading to NADPH production and steer away from NADH formation (Mailloux 2011, Lemire 2010b; Singh 2008). Metabolic cycles have been shown in *P. fluorescens* to balance the NADH:NADPH ratios and the enzymes involved were investigated. As expected, the cycle was up-regulated in the H$_2$O$_2$-stressed cells in comparison to the control (figure 44).

**Figure 57.** NADPH production is ensured by a NADH sequestering and NADP producing cycle in *P. fluorescens*. This metabolic network is promoted by NADK.

PC, MDH, ME, NADK and NADH oxidase were activated under oxidative stress to maintain a steady supply of NADPH to the cell (figure 57). In addition to this cycle, glucose metabolism also contributed to the production of NADPH during growth in an oxidative environment. In *P. fluorescens*, the main glucose metabolizing pathway is
the Entner-Doudoroff pathway (ED pathway) which transits via 6-phosphogluconate (6PG) and through a two step reaction makes pyruvate and glyceraldehyde-3-phosphate. The classical glycolysis pathway (Embden-Meyerhof pathway) is not functional in this bacterium due to the lack of the gene for phosphofructokinase (Buch, 2010; Paulsen, 2005). Leading to the production of 6PG in the stressed cells is the periplasmic NADPH producing GDH. This enzyme is also more active in menadione stressed cells and returning the stressed cells to normal media (and vice versa) reversed this change (figure 46, table 11). In addition to GDH, other enzymatic sources of NADPH showed increased activity in the stress, notably G6PDH and 6PGDH (figure 47). Involvement of these three enzymes (GDH, G6PDH and 6PGDH) shows a preference for the pentose phosphate pathway (PPP) rather than the ED pathway in the cells exposed to H₂O₂. The control cells preferentially metabolized glucose via HK followed by the ED pathway.

The preference for the PPP in stressed cells was confirmed by HPLC analysis. Formation of G3P and F6P was observed after only 10 min in the stressed cells during 6PG soluble CFE reactions whereas the control cells took longer to accumulate F6P (figure 48). Soluble CFE experiments with the transketolase inhibitor, oxythiamine (Liu, 2010), helped confirm the increased involvement of the PPP in the stressed cells (personal observation). The involvement of the PPP in NADPH production was further confirmed by the control culture increase in GPI activity. This enzyme converts G6P into F6P, an important precursor to cell wall components like glucosamine derivatives.
(Foley, 2008). The stressed cells showed a decreased GPI activity in order to avoid redundancy since the PPP yields F6P (figure 45). As expected, the glucose metabolizing pathways in the peroxide stressed cells is geared towards NADPH production which is promoted via GDH and the PPP (figure 58).

A)

**Control cells**

```
Glucose
  ↓
HK/G6PDH
  ↓
6PG
  ↓
ED pathway/glycolysis
  ↓
Pyruvate
  ↓
PDH
  ↓
Oxidative phosphorylation
```

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Figure 58. Global view of glucose metabolism in *P. fluorescens* in A) control conditions and in B) H$_2$O$_2$ stress condition. Glucose metabolizing pathways in the H$_2$O$_2$-stressed cells maintains NADPH production with the help of GDH and the PPP (G6PDH and 6PGDH). The PPP leads to pyruvate that is accumulated to scavenge ROS. ATP supply is ensured by the PEPS/AK system and acetate metabolism is increased.
4.5. Conclusion

Through metabolomics and proteomic studies, novel stratagems in survival during H₂O₂ insult were investigated in *P. fluorescens*. These cells summoned metabolic shifts leading to the accumulation of pyruvate, an antioxidant molecule, in order to deal with the threat of an oxidative environment. The resulting acetate formation from the non-enzymatic decarboxylation reaction led to an increase in acetate metabolism through AcK, further proving the ROS scavenging potential of the α-ketoacid pyruvate. As oxidative phosphorylation was shut down to limit ROS generation, SLP was called upon to meet the energy demands of the stressed cells. As PEPS and AK worked in concert to ensure adequate ATP yields, GDH and the PPP were employed to sustain NADPH and pyruvate production. As work in this area continues, alternate phosphotransfer enzymes along with high energy storage molecules will have to be investigated. To support the findings of this work, the change in enzyme activity seen between the control and the stressed cells should be correlated with protein expression/modification. The role of metabolites like pyruvate and acetate, two moieties that are tightly modulated during H₂O₂ stress, in translational and transcriptional regulation needs to be explored. Acetate accumulation due to ROS toxicity may well play a role in intracellular signaling and the manipulation of acetate gradient is key to genetic and epigenetic-driven processes. Protein acetylation and RNA acetylation might play a role in orchestrating the adaptations that allow *P. fluorescens* to thrive in oxidative environments.
This work adds two seminal concepts in molecular understanding of the adaptation to oxidative stress. Eliminating ROS is just one aspect of this story, a narrative that has been the mainstay of this biochemical exploration in the scientific literature. Here, the role of metabolism in generating pyruvate, a potent antioxidant, and in fulfilling the energy needs of the organism despite an ineffective respiratory chain has been revealed. It is quite possible that these metabolic networks may be operative in other living systems.

**Figure 59.** An integrated portrait of molecular adaptations to H$_2$O$_2$ in *P. fluorescens*. (green arrow = increase; red arrow = decrease).
5. References


DeCoursey, T. (2010) Voltage-gated proton channels find their dream job managing


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