Chapter 5

Alteration in the central carbon metabolism in chromomycin deletion mutant - JP1

In the present chapter, we attempted to probe possible reasons for one of the pleiotropic phenotype of lack of growth of JP1 mutant on chemically defined minimal media with glucose as sole carbon source.

5.1 Introduction

5.1.1 Influence of oxidative stress on central carbon metabolism

Besides adaptive responses such as activation of redox responsive transcriptional regulators and expression of antioxidant defense (chapter 4), metabolic plasticity allows rewiring of central carbon metabolism to overcome the oxidative stress. The reduced nicotinamides, NADH and NADPH play essential role in controlling cellular redox status. It is a well-documented fact that NADH fuels ATP production via oxidative metabolism and NADPH is required to maintain reducing environment for cellular processes. The study on soil bacteria, *Pseudomonas fluorescens* and *E. coli* signifies that under oxidative stress condition cellular amounts of NADH decreases and that of NADPH increases (1, 2). This is accomplished via three main mechanisms (i) increasing activity of NADPH generating enzymes and decreasing activity of NADH generating enzymes (1-3), (ii) decreasing NADP phosphatase activity by upregulation of NAD kinase (2) and (iiii) Using metabolic modules which allows NADH utilization, NADPH production and ATP generation by substrate level phosphorylation instead of oxidative phosphorylation (1-5). The metabolic rewiring in response to oxidative stress is well studied in *P. fluorescens* which includes decreasing flux through electron transport chain (ETC) – the main source for generation of ROS by curtailing NADH production, increasing flux through NADPH generating pathway and generation of ATP and other important metabolites through other routes as Fe containing enzymes of TCA and ETC are highly sensitive to ROS.

The metabolic reprogramming occurring in response to oxidative stress is universal which is seen both in prokaryotes and eukaryotes. It is reported that metabolic reprogramming occurs in *E. coli* in defense and in adaptation to oxidative stress induction. There is an increase in flux through phosphoenolpyruvate carboxykinase (PEPCK), phosphoenolpyruvate carboxylase (PEPC), malic enzyme (MEZ) and serine hydroxymethyltransferase (SHMT) in *E. coli* in response to paraquat stress. This increase in flux is in response to increased demands for reducing

power in oxidative stress condition (6). In *Staphylococus aureus*, TCA cycle is inactivated in response to ROS stress to curtail production of NADH (7). In *P. fluorescence* glycoxylate shunt pathway is operated in response to oxidative stress to avoid TCA (1).

5.1.2 Combating ROS via ketoacid accumulation

It is reported in the case of *P. fluorescens*, *E. coli*, hepatic and astrocitic cell lines that ROS induction can lead to ketoacid accumulation within and outside the cells. Pooling ketoacids is thought to be novel mechanism of metabolism in response to oxidative stress (8). In *P. fluorescens* the ketoacids such as ketoglutarate, pyruvate and glyoxlate are found to be accumulated during ROS stress. In the presence of oxidizing agents, these ketoacids undergo non enzymatic decarboxylation resulting in generation of succinate, acetate and formate respectively. Upon ROS induction, the level of (i) ketoglutarate is found to increase in *P. fluorescens* via modulation of TCA cycle enzymes (by upregulation of isocirate dehydrogenase and downregulation of ketoglutarate dehydrogenase (KGDH)) and by deamination of glutamate (by increase in glutamate dehydrogenase activity and decreasing ketoglutarate dehydrogenase activity); (ii) pyruvate is increased (by enhancing activities of pyruvate kinase and PEP synthase, diminishing activity of pyruvate dehydrogenase. It is also increased by deamination of aspartate (by the coordinated increase in activity of aspartate transaminase, phosphoenolpyruvate carboxy kinase and the PEP dephosphorylating enzymes like pyruvate phosphate dikinase); (iii) glyoxylate is increased (by increased activity of isocitratelyase coupled to the diminished activity of KGDH). Glyoxylate is generated via an increased activity of glycine transaminase and glycine dehydrogenase (8).

There are several studies showing that the ketoacids - pyruvate and ketoglutarate can attenuate metabolically generated oxidative stress. In case of *E. coli* cold shock and starvation stress led to the condition ‘viable but non –culturable state’, which was reversed by providing pyruvate or KG externally in the medium (9). Thus ketoacids are generated as scavengers for ROS.

5.1.3 Global metabolic changes in different organisms in response to oxidative stress

In response to oxidative stress, there is alteration in activities of transcriptional factors leading to global changes in protein activities and metabolic fluxes. In order to study systematically, the global responses to oxidative stress, where alterations in expression of thousands of genes and proteins are simultaneously measured proteomic and gene expression approach is usually undertaken. Greenberg *et. al.*, monitored dramatic changes in protein composition on 2-dimensional PAGE in *E. coli* treated with redox cycling agents such as menadione and paraquat (PQ) (generates superoxide radicals at sublethal concentration). Interestingly, they showed that, the redox cycling agents elicit similar cellular physiology as induced by H_2O_2. The intracellular superoxide generated by these agents also triggered induction of 33 other proteins not induced by H_2O_2 stress, which includes heat shock proteins, endonuclease IV, Mn-SOD and glucose 6-phosphate dehydrogenase (10, 11).

The whole genome transcriptional profiling of *E. coli* in response to superoxide generating PQ stress revealed modulation of around 112 genes in response to PQ stress. The adaptive changes include modulation of genes for replenishing reducing potential, iron transport and storage, sugar and amino acid transport, detoxification, protein modification, osmotic protection and peptidoglycan synthesis. PQ acts as 'double edged sword' in sense that intracellularly, it is reduced at expense of NADPH and following reduction, generates flux of superoxide. This in turn decreases levels of NADPH –a reducing power inside the cells. In order to replenish reducing power, G6PDH is activated which is the first enzyme of pentose phosphate pathway. Simultaneous inactivation of TCA cycle enzymes - fumarase C and aconitase contributes to reduction of NAD^+. The activation occurs at transcriptional level in *soxRS* dependent manner. Additionally, PQ stress also activated genes coding for proteins involved in sugar transport, amino acid transport and degradation, glycolysis and TCA cycle. PQ stress also activates genes coding ribosomal proteins which increase translational capacity in order to counterbalance faster turnover of proteins due to oxidative damage and increased degradation (12).

The adapative changes in gene expression in response to H_2O_2 stress is also observed in *Saccharomyces cerevisiae*. Godon *et. al,* illustrated by two dimensional

gel electrophoresis that H_2O_2 responsive targets are heat shock proteins, proteins involved in scavenging reactive oxygen species with simultaneous slowdown of protein biosynthesis pathway and stimulation of protein degradation pathway. Remarkably, resetting of carbohydrate metabolism is also observed. Genome wide characterization of H_2O_2 stimulon in *S. cerevisiae* indicated redirection of carbon flux towards regeneration of NADPH at expense of glycolysis (13).

A model has been proposed based on time series of microarray design in which a SoxRS-dependent and independent dynamic transcriptional networks responding to superoxide stress is divulged constituting 226 protein coding genes and small RNA sequences (14).

The metabolic flux analysis in *E. coli* under PQ induced oxidative stress suggested that the metabolic flux of glycolytic pathway is directed towards pentose phosphate pathway in response to PQ stress. Moreover, in response to oxidative stress acetate production was found to increase with decreased TCA flux and increased glyoxylate shunt. The global flux changes affected NADPH:NADH ratio which was found to be augmented with accumulation of α- ketoglutarate (15).

The carbon flux distributions were studied by Coze F. *et. al.*, in two *Streptomyces coelicolor* A3 (2) strains: the wild type M145 and its derivative mutant M1146, in the latter, gene clusters encoding the four main antibiotic biosynthetic pathways- Calcium-Dependent Antibiotic (CDA), actinorhodin (ACT), undecylprodigiosin (RED) and methylenomycin (MET) were deleted. They found that in M1146, there is an increased flux through pentose phosphate pathway and decreased flux through TCA. The redistribution of flux favored increased NADPH generation to maintain cellular redox homeostasis. It is proposed that lack of antibiotic actinorhodin production in M1146 might lead to increase in intracellular oxidative stress, which further impeded TCA cycle, ATP synthesis, biomass generation and glucose consumption (16).

In summary, different studies indicate that oxidative stress elicits global transcriptional changes affecting protein levels ultimately leading to altered metabolic flux. Thus organism rewires its metabolism in order to circumvent deleterious effects of oxidative stress. Longevity of an organism is compromised if the rewiring is not

sufficient enough to counter oxidative stress as is evident in the results described in this chapter.

Since one of the pleiotropic phenotype of chromomycin non-producer mutant is the complete absence of growth on minimal glucose medium, we tried to investigate the apparently provocative inference that the secondary metabolism affects primary metabolism. We demonstrate the effect is indeed real though indirect and that the inability to synthesize the secondary metabolite, chromomycin causes an increase in oxidative stress in the mutant which in turn reconfigures the central carbon metabolism resulting in inability of the mutant to utilize glucose as the sole carbon source. We proved (in chapter 6) that chromomycin indeed possesses antioxidant properties and the glycolytic and TCA cycle enzymatic perturbations are reversed by extracellular supplementation of sub-lethal concentration of chromomycin.

5.2 Results

5.2.1 JP1 fails to grow on mineral media with glucose as the sole carbon source

Chromomycin non-producer mutant JP1 did not grow on mineral minimal medium containing glucose as the sole carbon source (Fig. 5.1). In the minimal medium (R4) used for culturing *Streptomyces*, the carbon source is glucose and proline, and the nitrogen source is proline. However, JP1 mutant grew equally well on minimal medium supplemented with 0.5% casaminoacids (CAA) with or without glucose (Fig. 5.1) or with the mixture of 0.5% of twenty amino acids, but not when CAA was less than 0.1%, indicating that the amino acids were utilized as a carbon source rather than meeting auxotrophic requirements.

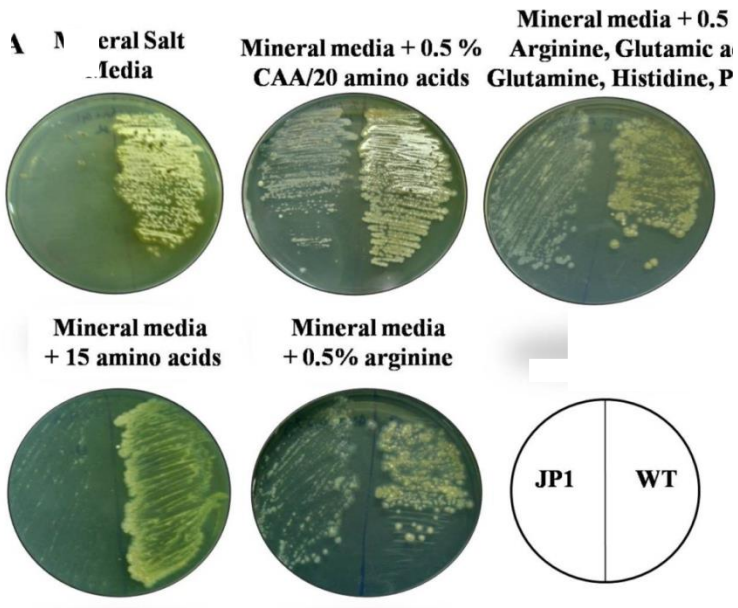

Figure 5.1 Growth of WT and JP1 was compared on mineral salt media / R4 media with supplementations as indicated (C source is glucose and proline, N source is proline and P source is potassium phosphate)

5.2.2 Glucose uptake defect is ruled out in JP1 mutant

First and foremost we tried to establish that there is no problem with glucose uptake by JP1 mutant. The results obtained by glucose estimation by GOD/POD kit (Reckon Diagnostics) are shown in Table 5.1. The results indicated that glucose uptake and metabolism is complete at the end of the growth in the JP1 as the concentration of glucose measured is undetectable.

Table 5.1 Glucose amounts measured by GOD/POD kit at start and end of the growth of WT and JP1.

Days	**Glucose amounts in mg/ml**	
	WT	JP1
1st day (at the time of inoculation)	2.4	2.4
2nd Day	0	0
4thDay	0	0

5.2.3 Oxidative stress in JP1 mutant reconfigures central carbon metabolism

5.2.3.1 The glycolytic phosphofructokinase (PFK) activity and expression are deficient in JP1

Glucose is metabolized primarily by glycolysis. The glucose flux through glycolysis and PPP has been estimated to be 70:30 (17). The flux is affected under oxidative stress. As a result of adaptation to oxidative stress, enhancement in the carbon flow into the PPP pathway is realized at the expense of glycolysis. 6-phosphofructokinase (PFK) is the important regulatory, rate limiting and the first committed enzyme of glycolysis, a reason for choosing PFK for measuring carbon flux through glycolysis (18). The functional equivalents of the *pfk* genes were decoded by comparing the genome sequence information of *S. flavisclerotic*us with other *Streptomyces* species.

5.2.3.2 Deciphering functional equivalents of *pfk* genes comparing genome sequence information of *S. flavisclerotic*us (Gene Bank Accession No. MAZZ00000000) and other *Streptomyces*

In Actinobacteria, two types of PFKs are found depending upon the phosphoryl donor i.e. ATP- and PP_i- (pyrophosphate) dependent. An uncommon ADP dependent PFK is limited to Archaebacteria. Family A of PFK is common in bacteria. ATP-PFKs carry out the irreversible reaction, regulated at the activity level by allosteric regulators like citrate and ATP/AMP (19) whereas PP_i-PFKs are not subject to any regulation. *S. flavisclerotic*us contains 3 PFKs and their identity was inferred from protein sequence identity/similarity of each of the three PFKs with those of *S. coelicolor* and also corroborated by finding the synteny of the flanking genes of each of the PFKs with those of *S. coelicolor*. The signature amino acids at positions 73(V/L), 76(D) and 104(G) that characterize the ATP-PFKs are found in PFK 1 and -3 in genes of *S. flavisclerotic*us. In the case of PP_i-PFK, signature amino acids at positions 73(T), 74(N) and 104(D) are conserved in PFK2 (20) and indeed found in the PFK2 of *S. flavisclerotic*us.

5.2.3.3 PFK activity measurement

We individually assayed the ATP-PFK and PP_i-PFK enzymes from cell-free extracts of the WT (and INT) and JP1 by the method described by Borodina *et. al.*, (17) . Importantly, the levels of the ATP-dependent enzyme (PFK3) were reduced by more than two-fold in JP1 than in the WT (and INT) (Fig. 5.2A), whereas the PP_i-dependent activity of PFK (PFK2) was undetectable.

5.2.3.4 Transcription analysis of *pfk* genes

Quantitation of RNA transcript amounts of each of the *pfk* genes by reverse transcriptase PCR demonstrated that the decrease in the PFK activity effect coincides with the reduced transcript levels of each of the two *pfk*s, *pfk2 and pfk3* (Fig. 5.2B). In contrast, the transcript of the second putative ATP-dependent *pfk1* was undetectable in both the WT (and INT) and JP1. Since the metabolic function of PP_i-dependent PFK in aerobic bacteria, including *Streptomyces,* is largely unknown (21), it is reasonable to assume that the decrease in the total PFK activity in the JP1 mutant is primarily due to the reduced transcript levels of *pfk3*.

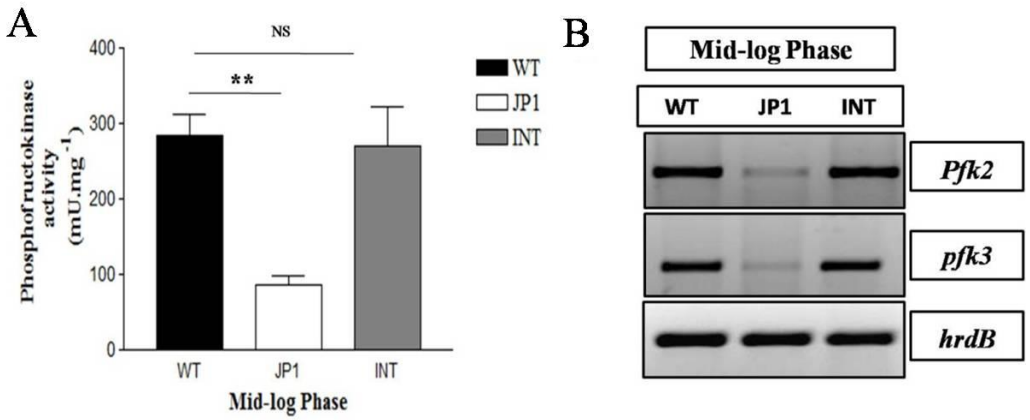

Figure 5.2 Activity and expression of phosphofructokinase (A) ATP-PFK activity was measured in mid-log phase of WT, JP1 and INT. Results are representative of three independent experiments; error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism3) for multiple comparisons and unpaired t-test; ** $p < 0.01$; (B) ATP-*pfk* (*pfk3*) and PPi-*pfk* (*pfk2*) mRNA levels were quantitated by semi quantitative RT-PCR of RNA extracted from cells of WT, INT and JP1 in mid-log phase. The transcription profiles are representative of three independent experiments**.**

5.2.3.5 Reduction in amounts of acetyl –CoA in JP1

The reduction in the channeling of glucose through glycolysis may have affected the levels of the end product acetyl-CoA accordingly, the amounts of this metabolite in JP1 were reduced two-fold relative to the WT, as measured by HPLC (Fig. 5.3).

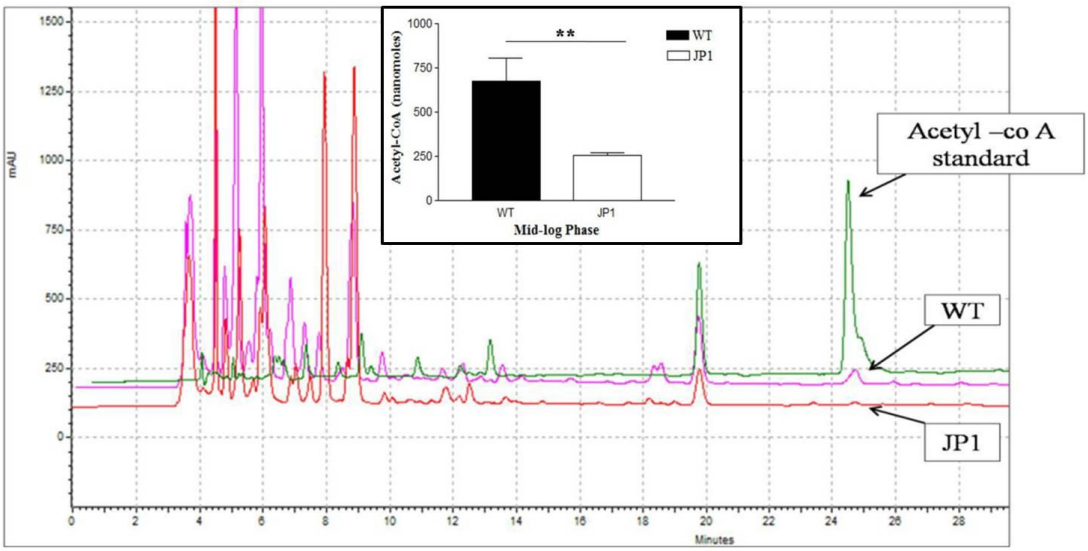

Figure 5.3 Acetyl Co-A estimation by HPLC - Intracellular levels of Acetyl Co-A in WT and JP1 as measured by HPLC in mid-log phase. Results are representative of three independent experiments; error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by unpaired t-test, ** $p < 0.01$.

5.2.3.6 Pentose Phosphate Pathway (PPP) in JP1 is upregulated.

The PPP is catabolic pathway and serves as alternative glucose oxidizing pathway for generation of NADPH required for reductive biosynthetic reactions. It converts fructose-6-phosphate to 6-phosphogluconate by glucose 6-phosphate dehydrogenase (G6PDH, *zwf*) and subsequently to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (6-PGDH) with each of these steps generating one NADPH equivalent of reducing power (19). The activities of both the enzymes of the PPP, G6PDH and 6-PGDH, were found to be ~ 2-fold higher in JP1 mutant in comparison to WT and INT (Fig. 5.4A; 5.4B).As a consequence, NADPH levels were

also found to be increased in JP1 by ~2.5-fold (Fig. 5.5) as compared to the WT (and INT).

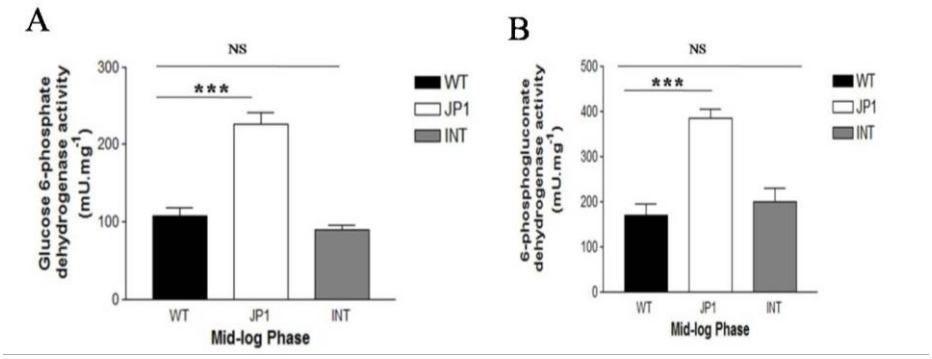

Figure 5.4 Measurement of enzyme activities of PPP: G6PDH activity and (D) 6-PGDH activity in the mid-log phase cells of WT, INT and JP1 (specific activity - units.mg^{-1} protein).

***p < 0.001

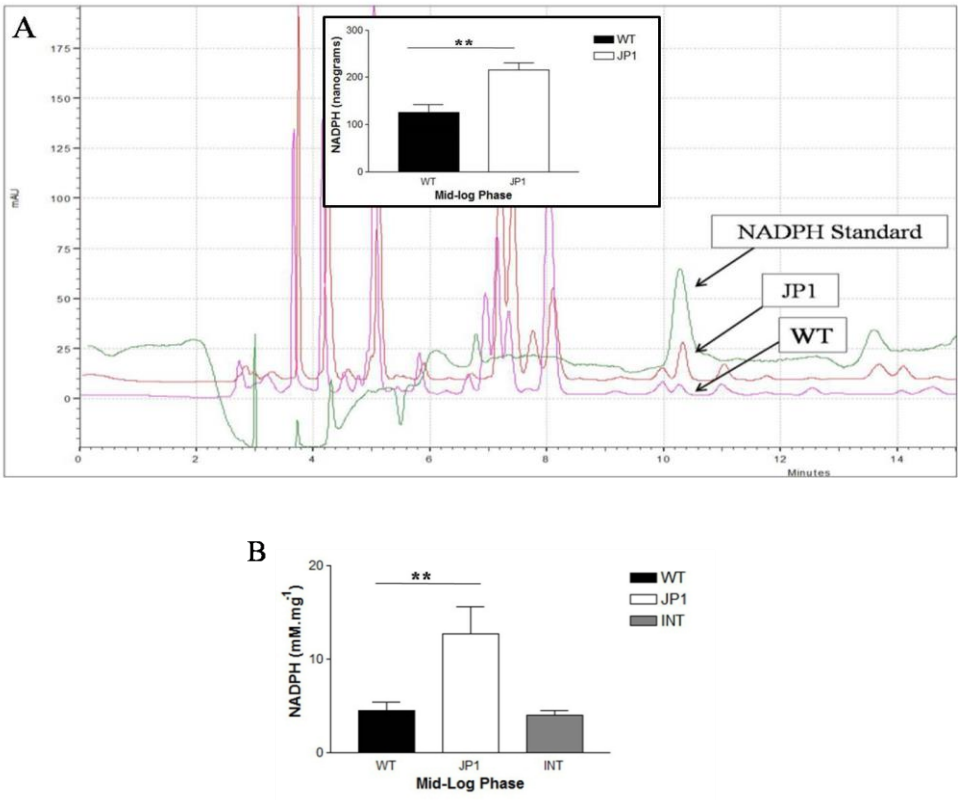

Figure 5.5 Analysis of intracellular levels of NADPH measured by (A) HPLC and (B) enzymatic cycling method in WT, INT and JP1 in mid-log phase of growth. ** $p < 0.01$.

5.2.3.7 Transcription analysis of glucose 6-phosphate dehydrogenase (*zwf*)

Expression profile of *zwf* in wild type and JP1 was compared. For distinguishing two highly similar (90%) sequences of *zwf* genes in the genome of *S. flaviscleroticus,* we designed primers by incorporating one (out of 3) of the differences in the nucleotide sequences of the *zwf* at the 3'end of the primer. The *zwf* transcripts amounts were consistently higher in the JP1 than in the WT. Thus transcriptional upregulation accounted for the enhancement of Zwf activity since the *zwf* transcripts' amounts were consistently higher in JP1 than in the WT (Fig. 5.6).

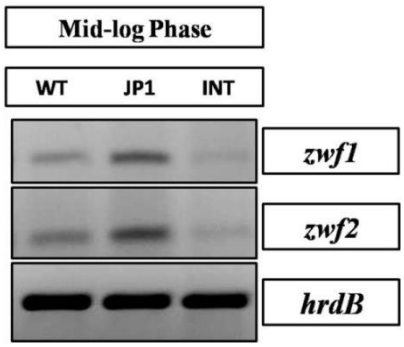

Figure 5.6 Expression of *zwf*: mRNA levels were quantitated of *zwf*1 and –2 by semi quantitative RT-PCR of RNA extracted from cells of WT, JP1 & INT in mid-log phase. The transcription profiles are representative of three independent experiments.

5.2.3.8 JP1 presents an impaired TCA cycle

To counter ROS generation, tricarboxylic acid cycle (TCA) enzymes are generally downregulated at different levels (22). For example, aconitase which contains iron-sulfur center becomes inactive under oxidative stress condition due to the oxidation by ROS of the prosthetic group [4Fe-4S] cluster containing an unprotected labile iron [Fe] atom (22, 23). Therefore, the enzyme activities of aconitase (Acn) and isocitrate dehydrogenase were monitored and were found to be reduced by two- to four-fold in JP1 compared to the WT (and INT) (Fig. 5.7). Since TCA cycle is the site for generation of ROS, the downregulation of its enzymes is consistent with the adjustments required to counter enhanced oxidative stress in the

JP1 mutant possibly slowing the growth of the mutant as a result. Consequently, the levels of α-KG (α –KetoGlutarate), the master regulator of the TCA cycle and a proposed antioxidant, were similarly reduced two-fold in JP1 as compared to WT (and INT) (Fig. 5.7). The JP1 mutant's growth was rescued only by the glucogenic amino acids that generate α-KG, including glutamate, glutamine, histidine, proline and arginine at concentrations that meet the carbon requirement of JP1 (Fig. 5.1). The combination of five amino acids was better at promoting growth than any individual amino acid. For reasons not yet determined, arginine supplementation is obligatory in combinations of two or three amino acids.

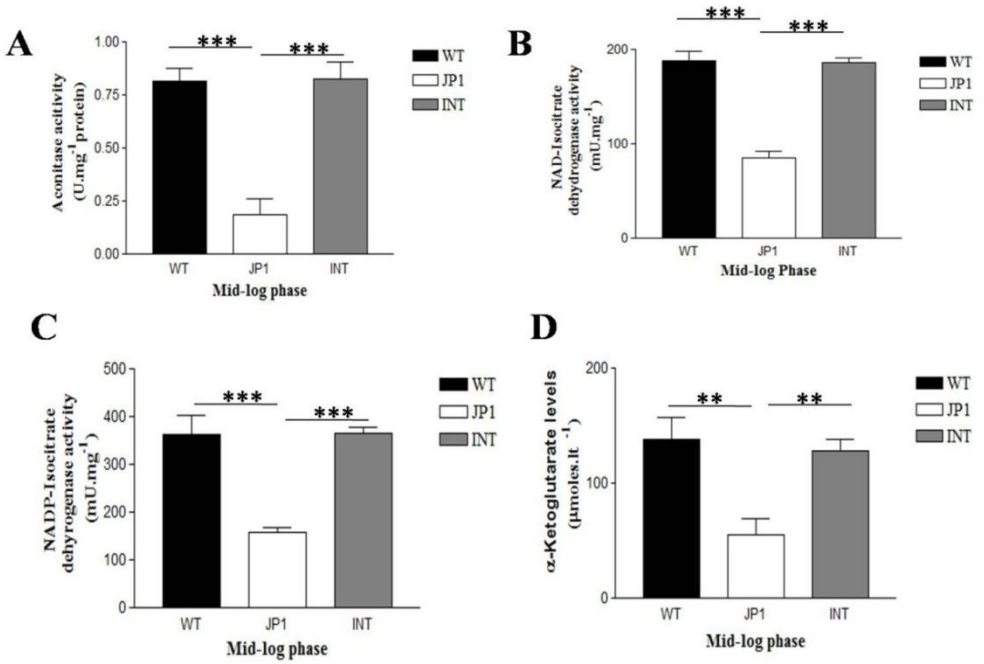

Figure 5.7 Measurement of TCA cycle enzyme activities: The TCA cycle enzyme activities were assayed in cells of WT, INT and JP1 grown to mid-log phase for (A) aconitase; (B) NAD - Isocitrate dehydrogenase (ICDH); (C) NADP - Isocitrate dehydrogenase (ICDH) (specific activity-units.mg^{-1} protein); (D) α-ketoglutatrate levels were measured in cells of WT, INT and JP1 by enzymatic method under the same conditions of growth. Results are representative of three independent experiments; error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism3) for multiple comparisons and unpaired t-test *$p < 0.05$, ** $p < 0.01$

5.2.4 *S. flavisclerotícus* preferentially utilizes amino acids as a carbon source over glucose and the effect of the preference on its tolerance to oxidative stress

S. flavisclerotícus displayed an inherent preference for amino acids as a carbon source over glucose (Fig. 5.8). The biomass yield in minimal media supplemented with casamino acids is double than in the minimal glucose media in accordance with its preference. When wild type cells were exposed to oxidizing agents like H_2O_2 and diamide which induce oxidative stress it was observed that the glucose grown culture was more vulnerable to inhibition by H_2O_2 and diamide than when grown in minimal media with CAA. Thus possibly, the preference of amino acids over glucose ultimately affects the tolerance of *S. flavisclerotícus* to oxidative stress as well (Table 5.2). NADPH, provides reductive environment which enables stressed cells to nullify reactive oxygen species (24). Thus the levels of NADPH were found to be 40% higher in cells grown in CAA than those grown in glucose (Table 5.2). This result is in agreement with the fact that the resistance to diamide is a direct measure of the intracellular NADPH levels (25).

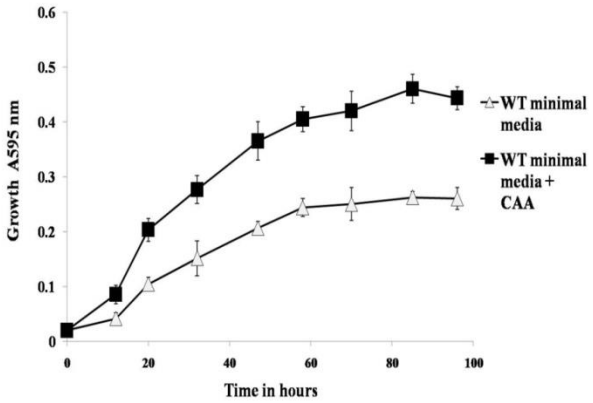

Figure 5.8 Inherent preference of WT for amino acids over glucose as a sole carbon source (A) Growth curve of WT/INT as measured by DNA estimation (A_{595} nm) in minimal medium with glucose as sole carbon source and in minimal glucose medium with 0.5 % CAA as carbon source.

Table 5.2 The effect of carbon content of the growth medium on tolerance to oxidative stress

Growth medium	Diamide		H_2O_2		NADPH levels ($mM.mg^{-1}$)
	Diameter (mm)	Surface area (mm^2)	Diameter (mm)	Surface area (mm^2)	
Minimal glucose	20.166 ± 0.28	319.255	20.26 ± 0.46	322.4	1.7 ± 0.15
Minimal glucose + Casamino acids	14.66 ± 0.57	168.86	10.33 ± 0.57	83.82	2.7 ± 0.17
The zone of inhibition of growth and area correspond to that formed by 0.5 M diamide and 0.9 M H_2O_2. The S.D. is determined from 3 independent experiments.					

5.3 Discussion

The inability of JP1 to utilize glucose as sole carbon source has been shown to be a result of the combined effects of inherent preference of *Streptomyces flavisclerotic*us for oxidative metabolism over glycolysis and the increased oxidative stress in the mutant reconfiguring the central carbon metabolism. *S. flavisclerotic*us exhibits an inherent preference for amino acids as a carbon source over glucose, which is also seen in the case of *Streptomyces coelicolor* and *Streptomyces fradiae* (26, 27). In *S. coelicolor,* the amino acid preference is assisted with robust oxidative metabolism and gluconeogenesis which is also true for *S. flavisclerotic*us. As a result, the glucose utilization by glycolysis is expected to be poor and unable to meet the demands of reducing power production under oxidative stress. In the case of *S. flavisclerotic*us, the carbon source preference is reflected in reduced yield of biomass in minimal media (almost half) as compared to that in minimal media supplemented with casamino acids. The amino acid catabolism and gluconeogenesis suffices for NADPH generation by PPP. Though *S. flavisclerotic*us and *S. coelicolor* are similar in possessing elevated gluconeogenesis and reduced glycolysis, unlike that in *S. flavisclerotic*us, the effect on biomass is not reported in case of *S. coelicolor* (28). Absence of growth on glucose containing media is not uncommon because *E. coli* and

Saccharomyces cerevisiae mutants affected in oxidative stress management are incapable of growth on minimal glucose media (29-31).

The carbon preference also affects tolerance of *S. flaviscleroticus* to oxidative stress. The increased susceptibility of wild type to H_2O_2 and diamide grown in minimal media with glucose as compared to that grown in CAA is clearly due to low NADPH levels in the former than in the latter growth medium. Indeed NADPH levels were found to be 40% less in glucose grown medium than CAA grown medium (Table 5.2). In order to validate the inference of oxidative metabolism in *S flaviscleroticus,* proteomic approach is required in *S. flaviscleroticus* too as in *S. coelicolor* (28).

Because of ROS stress, the pentose phosphate pathway in JP1 is upregulated which becomes apparent by increase in the activity of G6PDH and 6PGDH (Fig. 5.4; 5.9). The increased activity is a consequence of upregulation of *zwf* transcription (Fig. 5.6; 5.9). The increased activities of PPP enzymes ultimately raises the NADPH levels in JP1 mutant by almost 2.5-fold which is consistent with the knowledge that under oxidative stress condition reducing agent NADPH is overproduced to maintain cellular redox condition. Glycolytic flux is diverted to PPP in order to combat oxidative stress by reduced activity of rate limiting and first committed step of glycolysis ATP-PFK. The reduction at the expression levels of *pfk* is reflected in its activity level as well. The carbon utilization through PPP requires functional PFK, intermediates of PPP cannot be metabolized through glycolysis to generate adequate amounts of ATP (Fig. 5.9). The imbalance in the reduced/oxidized nucleotide pool as a consequence of NADPH overproduction could be detrimental to the growth, as is the case of *E. coli* (32, 33).

The enzymes of TCA cycle mainly having metal centers are generally downregulated to counter ROS (22, 23). Accordingly, levels of TCA cycle enzymes like aconitase, NAD/NADP IDH and metabolite α-KG found to be low in JP1 mutant (Fig. 5.9). α-KG is master regulator of TCA cycle and is a proposed antioxidant. There is a clear demonstration of involvement of α-KG in ROS detoxification in prokaryotes (34). As anticipated, the JP1 mutants' growth can be rescued by providing glucogenic amino acids - glutamate, glutamic acid, arginine, histidine and proline at concentration required to meet carbon needs. Thus, the deficiency of α-KG

could also be linked to increased oxidative stress in JP1. Notably, the catabolism of proline, arginine, glutamate, glutamine, and histidine support the growth of the JP1 mutant that generate α-KG, while proline catabolism has been shown to influence oxidative stress resistance in *E. coli* and different organisms (35). Thus, alterations in the TCA cycle enzymes and the levels of the metabolite α-KG can also indicate oxidative stress adjustments. We therefore postulate that the growth of JP1 could be limited by the availability of α-KG. None of the remaining 15 amino acids were able to support the growth of JP1 on mineral media (Fig. 5.1).

Taken together, the metabolic defects of the JP1 mutant are compatible with the proposal of increased oxidative stress and recapitulate some important manifestations of adaptive responses to oxidative stress. The strategy of enhancing the production of reducing power, NADPH, as a consequence of the upregulation of the PPP and the downregulation of glycolysis (Fig. 5.9), commonplace in yeast and higher organisms, has not been documented in bacteria. Interestingly, the JP1 mutant seems to resemble eukaryotes in this regard.

Deletion of secondary metabolite genes for all the three antibiotic cluster (RED, ACT and CDA) has been found to affect the carbon metabolism in *S. coelicolor*. The preference for amino acids as carbon source in *S. coelicolor* (wild type M145) is clearly due to the abundance of proteome of TCA cycle over that of glycolysis. A higher oxidative metabolism, more active gluconeogenesis and a reduced glycolytic flux in *S. coelicolor* has been proposed to result in a reduced flux through the PPP and characterizes the carbon flux distribution in the antibiotic producer *S. coelicolor* M145. In the deletion mutant M1146 of *S. coelicolor* lacking the four major antibiotics CDA, ACT, RED and methylenomycin, there is higher flux through pentose phosphate pathway (PPP) which generates more NADPH for biomass production, to maintain redox homeostasis and other cellular processes like TAG (TriAcylGlycerol) biosynthesis. Incidentally, high NADPH also assists in coping oxidative stress in the cell. Moreover, there was low glucose uptake and flux through Kreb cycle was also low. This is yet an evidence for secondary metabolism affecting primary metabolism (27).

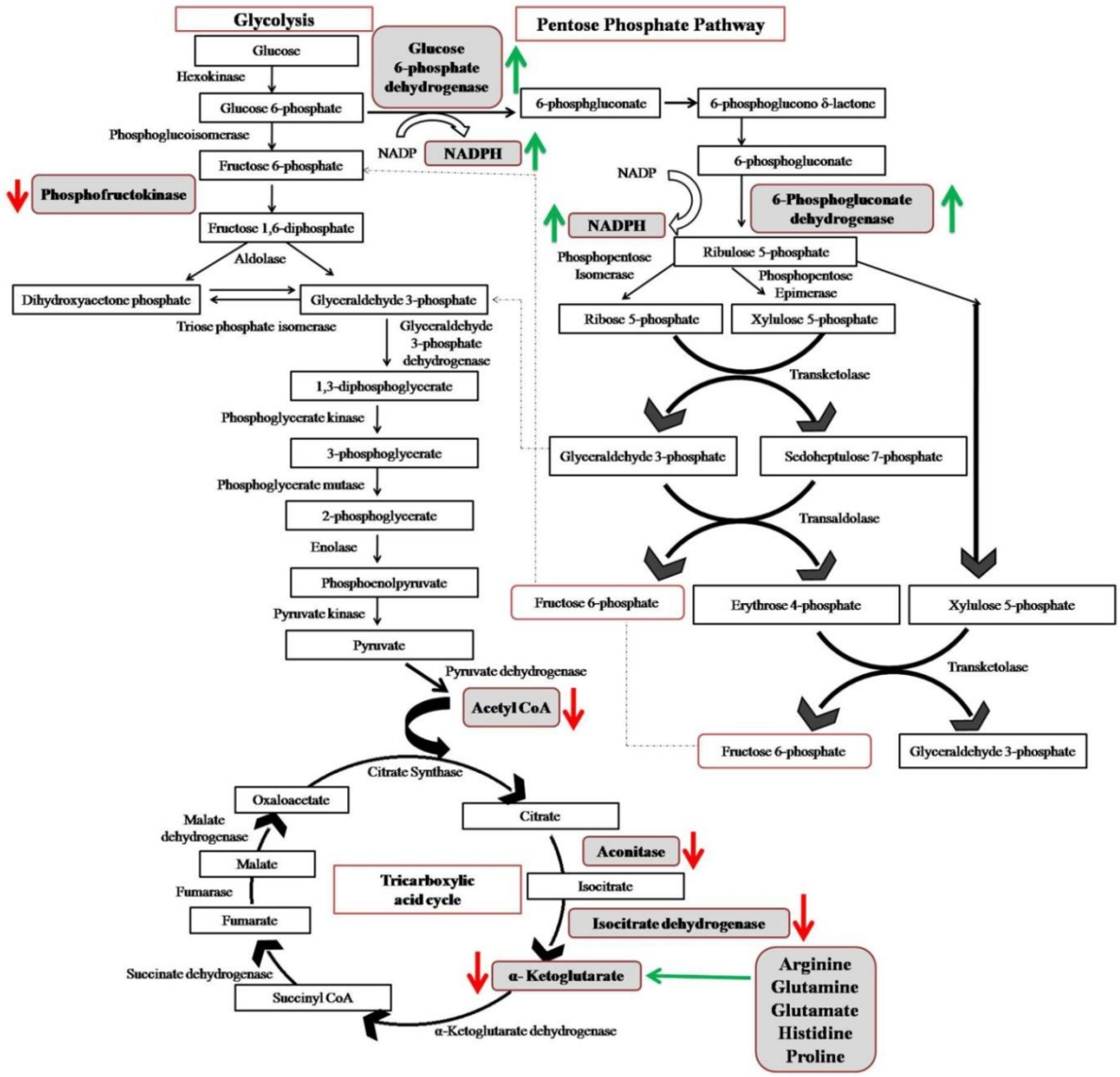

Figure 5.9 Schematic diagram of oxidative stress-effected changes in the central carbon metabolism in the JP1 mutant - The enhancement and reduction in the levels of enzyme activities (highlighted in green and red arrows respectively) and the metabolites (highlighted in grey box) as measured in the JP1 mutant compared to wild type. The dotted lines indicate intermediates of PPP pathway re-entering into the glycolysis.

5.4 References

1. Mailloux RJ & Harper M-E (2011) Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radical Biology and Medicine* 51(6):1106-1115.
2. Singh R, Lemire J, Mailloux RJ, & Appanna VD (2008) A novel strategy involved anti-oxidative defense: the conversion of NADH into NADPH by a metabolic network. *PLoS One* 3(7):e2682.
3. Mailloux RJ, *et al.* (2007) The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. *PloS one* 2(8):e690.
4. Aldarini NSS (2017) Glutamine metabolism and energy homeostasis during oxidative stress. (Laurentian University of Sudbury).
5. Thomas SC, Alhasawi A, Auger C, Omri A, & Appanna VD (2016) The role of formate in combatting oxidative stress. *Antonie van Leeuwenhoek* 109(2):263-271.
6. Shen T, *et al.* (2013) Metabolic flux ratio analysis and multi-objective optimization revealed a globally conserved and coordinated metabolic response of E. coli to paraquat-induced oxidative stress. *Molecular BioSystems* 9(1):121-132.
7. Somerville GA, *et al.* (2003) Synthesis and deformylation of Staphylococcus aureus δ-toxin are linked to tricarboxylic acid cycle activity. *Journal of bacteriology* 185(22):6686-6694.
8. Lemire J, Alhasawi A, Appanna V, Tharmalingam S, & Appanna V (2017) Metabolic defence against oxidative stress: the road less travelled so far. *Journal of applied microbiology* 123(4):798-809.
9. Mizunoe Y, Wai SN, Takade A, & Yoshida S-i (1999) Restoration of culturability of starvation-stressed and low-temperature-stressed Escherichia coli O157 cells by using H2O2-degrading compounds. *Archives of Microbiology* 172(1):63-67.
10. Greenberg JT & Demple B (1989) A global response induced in Escherichia coli by redox-cycling agents overlaps with that induced by peroxide stress. *J Bacteriol* 171(7):3933-3939.
11. Greenberg JT, Monach P, Chou JH, Josephy PD, & Demple B (1990) Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli. *Proceedings of the National Academy of Sciences* 87(16):6181-6185.
12. Pomposiello PJ, Bennik MH, & Demple B (2001) Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate. *J Bacteriol* 183(13):3890-3902.
13. Godon C, *et al.* (1998) The H2O2 stimulon in Saccharomyces cerevisiae. *J Biol Chem* 273(35):22480-22489.
14. Blanchard JL, Wholey W-Y, Conlon EM, & Pomposiello PJ (2007) Rapid changes in gene expression dynamics in response to superoxide reveal SoxRS-dependent and independent transcriptional networks. *PloS one* 2(11):e1186.
15. Rui B, *et al.* (2010) A systematic investigation of Escherichia coli central carbon metabolism in response to superoxide stress. *BMC systems biology* 4(1):122.
16. Coze F, Gilard F, Tcherkez G, Virolle M-J, & Guyonvarch A (2013) Carbon-flux distribution within Streptomyces coelicolor metabolism: a comparison between the actinorhodin-producing strain M145 and its non-producing derivative M1146. *PLoS One* 8(12):e84151.

17. Borodina I, *et al.* (2008) Antibiotic overproduction in Streptomyces coelicolor A3 (2) mediated by phosphofructokinase deletion. *Journal of Biological Chemistry* 283(37):25186-25199.
18. Berg JM, Tymoczko JL, & Stryer L (2002) *Biochemistry 5th edition, 2002* (WH Freeman. Available online through the NCBI Bookshelf: link).
19. Berg JM, *et al.* (2002) Biochemistry: International version (hardcover). (WH Freeman).
20. Siebring J (2010) *The phosphofructokinases of Streptomyces coelicolor A3 (2)*.
21. Mertens E (1991) Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? *FEBS letters* 285(1):1-5.
22. Nyström T (2004) Stationary-phase physiology. *Annu. Rev. Microbiol.* 58:161-181.
23. Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 77:755-776.
24. Singh R, Mailloux RJ, Puiseux-Dao S, & Appanna VD (2007) Oxidative stress evokes a metabolic adaptation that favors increased NADPH synthesis and decreased NADH production in Pseudomonas fluorescens. *Journal of bacteriology* 189(18):6665-6675.
25. Borodina I, *et al.* (2008) Antibiotic overproduction in Streptomyces coelicolor A3 2 mediated by phosphofructokinase deletion. *J Biol Chem* 283(37):25186-25199.
26. Romano AH & Nickerson WJ (1958) Utilization of amino acids as carbon sources by Streptomyces fradiae. *Journal of bacteriology* 75(2):161.
27. Millan-Oropeza A, *et al.* (2017) Quantitative Proteomics Analysis Confirmed Oxidative Metabolism Predominates in Streptomyces coelicolor versus Glycolytic Metabolism in Streptomyces lividans. *J Proteome Res* 16(7):2597-2613.
28. Millan-Oropeza A, *et al.* (2017) Quantitative proteomics analysis confirmed oxidative metabolism predominates in Streptomyces coelicolor versus glycolytic metabolism in Streptomyces lividans. *Journal of proteome research* 16(7):2597-2613.
29. Seaver LC & Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli. *J Bacteriol* 183(24):7173-7181.
30. Benov L & Fridovich I (1999) Why Superoxide Imposes an Aromatic Amino Acid Auxotrophy onEscherichia coli THE TRANSKETOLASE CONNECTION. *Journal of Biological Chemistry* 274(7):4202-4206.
31. Liu X, *et al.* (1992) Yeast lacking superoxide dismutase. Isolation of genetic suppressors. *Journal of Biological Chemistry* 267(26):18298-18302.
32. Auriol C, Bestel-Corre G, Claude JB, Soucaille P, & Meynial-Salles I (2011) Stress-induced evolution of Escherichia coli points to original concepts in respiratory cofactor selectivity. *Proc Natl Acad Sci U S A* 108(4):1278-1283.
33. Canonaco F, *et al.* (2001) Metabolic flux response to phosphoglucose isomerase knock-out in Escherichia coli and impact of overexpression of the soluble transhydrogenase UdhA. *FEMS Microbiol Lett* 204(2):247-252.
34. Lemire J, *et al.* (2010) Histidine is a source of the antioxidant, alpha-ketoglutarate, in Pseudomonas fluorescens challenged by oxidative stress. *FEMS Microbiol Lett* 309(2):170-177.
35. Zhang L, Alfano JR, & Becker DF (2015) Proline metabolism increases katG expression and oxidative stress resistance in Escherichia coli. *Journal of bacteriology* 197(3):431-440.