



# Phospho-transfer networks and ATP homeostasis in response to an ineffective electron transport chain in *Pseudomonas fluorescens*



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## ABSTRACT

Although oxidative stress is known to impede the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, the nutritionally-versatile microbe, *Pseudomonas fluorescens* has been shown to proliferate in the presence of hydrogen peroxide ( $H_2O_2$ ) and nitrosative stress. In this study we demonstrate the phospho-transfer system that enables this organism to generate ATP was similar irrespective of the carbon source utilized. Despite the diminished activities of enzymes involved in the TCA cycle and in the electron transport chain (ETC), the ATP levels did not appear to be significantly affected in the stressed cells. Phospho-transfer networks mediated by acetate kinase (ACK), adenylate kinase (AK), and nucleoside diphosphate kinase (NDPK) are involved in maintaining ATP homeostasis in the oxidatively-challenged cells. This phospho-relay machinery orchestrated by substrate-level phosphorylation is aided by the up-regulation in the activities of such enzymes like phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), and phosphoenolpyruvate synthase (PEPS). The enhanced production of phosphoenolpyruvate (PEP) and pyruvate further fuel the synthesis of ATP. Taken together, this metabolic reconfiguration enables the organism to fulfill its ATP need in an  $O_2$ -independent manner by utilizing an intricate phospho-wire module aimed at maximizing the energy potential of PEP with the participation of AMP.

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## 1. Introduction

Oxidative stress is a major hazard that all aerobic organisms have to face. The reactive oxygen species (ROS), such as superoxide radical ion ( $O_2^-$ ) and peroxynitrite ( $OONO^-$ ), generated as a consequence of metabolic processes tend to react with the essential machinery designed to sustain life [1,2]. The result is a breakdown of normal energy production, oxidation of lipids, proteins, and perturbation of genetic information [3–7]. To counter these noxious effects of ROS, numerous organisms have developed effective strategies aimed at detoxifying these harmful entities. This includes metabolites like glutathione, which in conjugation with the enzyme glutathione reductase (EC 1.8.1.7), can be used to effectively neutralize ROS and reactive nitrogen species (RNS) [8–10]. The enzymes catalase (EC 1.11.16) and superoxide dismutase (EC 1.15.1.1) convert hydrogen peroxide ( $H_2O_2$ ) and superoxide into innocuous species [11]. The production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a powerful anti-

oxidant, has been reported to be up-regulated during oxidative challenge [12–14]. Enzymes involved in its production, such as NADP<sup>+</sup>-dependent isocitrate dehydrogenase (ICDH-NADP<sup>+</sup>, EC 1.1.1.42), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) are activated in systems challenged by oxidative stress [15]. The role of exopolysaccharides and oxalate in mitigating oxidative stress has also been reported [16–18]. Recently, studies showing the participation of ketoacids in combatting oxidative stress present a new approach to detoxify the noxious radicals. Metabolic reprogramming resulting in the production of pyruvate,  $\alpha$ -ketoglutarate, and glyoxylate have been shown to contribute to the reduction of intracellular oxidative tension [14,19–22]. These molecules neutralise these harmful oxidative moieties, with the concomitant formation of acetate, succinate, and formate respectively. These carboxylic acids can also contribute to the energy budget and are involved in signalling processes and in the production of reducing factors [23–25].

Although ATP production via oxidative phosphorylation is severely disrupted during oxidative stress, numerous organisms are known to survive this challenge. Studies have shown that this may be related to the up-regulation of enzyme systems responsible for

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the formation of ATP by substrate-level phosphorylation [26]. Metabolic networks such as glycolysis that help generate phosphoenolpyruvate (PEP), a high energy compound are promoted. PEP can be readily converted into ATP with the aid of either pyruvate kinase (PK, EC 2.7.1.40), pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1) or phosphoenolpyruvate synthase (PEPS, EC 2.7.9.2). The former utilizes ADP as a cofactor while the latter two enzymes invoke the participation of AMP [27]. We have recently demonstrated a critical role for a metabolon involving citrate lyase (EC 4.1.3.6), phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and PPDK in ATP homeostasis in *Pseudomonas fluorescens* subjected to nitrosative stress [26]. This microbe affords an interesting model to study metabolic adaptation due to its nutritional versatility and its ability to proliferate in diverse environments. However, the efficacy of this process is dependent on the networks involved in siphoning the high-energy phosphate and generating AMP, events that further fuel the synthesis of ATP. In this study, the phospho-transfer networks that render this ATP-forming machine effective have been elucidated. The participation of adenylate kinase (EC 2.7.4.3), acetate kinase (EC 2.7.2.1), phosphate acetyltransferase (EC 2.3.1.8), acetyl CoA synthetase (EC 6.2.1.1) and nucleoside diphosphate kinase (EC 2.7.4.6) in ensuring the efficiency of the energy machine by transmitting the high energy phosphate from ATP to a variety of substrates has been shown. This phospho-relay process appears to be independent of carbon source the organism was cultured in. The interplay in the homeostasis of nucleotides and the significance of metabolic reprogramming in mitigating the inadequate ATP synthesis via oxidative phosphorylation are discussed.

## 2. Material and methods

### 2.1. Microbial growth conditions and isolation of cellular fractions

The bacterial strain *Pseudomonas fluorescens* 13525 was obtained from American Type Culture Collection (ATCC) and grown in a mineral medium containing (per liter of deionized water)  $\text{Na}_2\text{HPO}_4$  (6.0 g);  $\text{KH}_2\text{PO}_4$  (3.0 g);  $\text{MgSO}_4$  (0.2 g) and  $\text{NH}_4\text{Cl}$  (0.8 g). Aspartate, citrate, glucose, xylose and fumarate (19 mM) were utilized as carbon sources and were introduced separately to the mineral media. Glycerol was added at a concentration of 10% when used as a carbon source. The inclusion of glycerol and xylose in this study extends the range of chemical environments where the microbe can proliferate under oxidative stress and is aimed at establishing the common metabolic response dedicated to the production of ATP, irrespective of the source of carbon.

Trace elements (1 mL) were added as previously described [28]. The pH of the culture media was adjusted to 6.8 with 2 N NaOH. Prior to inoculation, the media were dispensed in 200 mL aliquots in 500 mL Erlenmeyer flasks and autoclaved. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (100  $\mu\text{M}$ ) or sodium nitroprusside (SNP) (5 mM) were sources of oxidative and nitrosative stress, respectively [19,26]. The amount of  $\text{H}_2\text{O}_2$  decreased over time. At logarithmic phase of growth 30% of the initial  $\text{H}_2\text{O}_2$  was evident, while at stationary phase of growth, the level was approximately 3%. To ensure that the organism was exposed to a similar oxidative challenge, another dose was added after the logarithmic phase of growth [20]. The stressors were added to the media following sterilization. The media were then inoculated with 1 mL of stationary-phase cells grown (400  $\mu\text{g}$  protein equivalent per mL) in their respective media and were incubated at 26 °C in a gyratory water bath shaker. Cells in control and  $\text{H}_2\text{O}_2$  or SNP cultures were isolated at similar growth phase by centrifugation at 11,000 rpm for 15 min at 4 °C. The supernatant was removed and the cellular pellets were washed with 0.85% NaCl and re-suspended in a cell storage buffer (pH 7.3) consisting of 50 mM Tris-HCl; 5 mM  $\text{MgCl}_2$  and 1 mM

phenylmethylsulfonylfluoride (PMSF). The cells were disrupted by sonication with the aid of a Brunswick sonicator, for 15 s, in 3 intervals. The cell free extracts (CFE) were obtained by centrifugation for 3 h at 50,000 rpm at 4 °C following the removal of the unbroken cells at 11,000 rpm. The Bradford assay was performed in triplicate to determine the protein concentration and bovine serum albumin (BSA) was used as the standard [29].

### 2.2. Regulation experiments

To confirm if the metabolic changes were indeed triggered by the oxidative stress, control cells were exposed to stress media and stressed cells were incubated in control media. To provide a proper comparison, these cells were obtained at the same growth phase. Following a 6 h incubation at 26 °C in a gyratory water bath, the cells were harvested as described before. The cellular fractions were isolated by centrifugation and assayed for select enzymatic activities. Only data with some representative carbon sources are shown.

### 2.3. Oxidized lipids

The levels of oxidized lipids in control and 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ -stressed cells were measured by using the thiobarbituric acid reactive species assay (TBARS) as described [30]. To assess the antioxidant property of pyruvate, 2 mg/mL protein equivalent of membrane CFE was incubated for 30 min in reaction buffer containing 2 mM pyruvate in the presence of sodium cyanide (5 mM), an inhibitor of oxidative phosphorylation. Negative controls were performed using 2 mg of membrane without pyruvate. These reactions were followed by HPLC and colorimetrically. The formation of acetate subsequent to the detoxification of ROS by pyruvate was measured.

### 2.4. Metabolite analysis

Metabolite levels were monitored using high performance liquid chromatography (HPLC). Cells were grown in control and  $\text{H}_2\text{O}_2$ /RNS-stressed conditions and isolated by centrifugation as described previously. The soluble fractions were boiled immediately for 10 min to precipitate proteins before analysis. This quenching technique enabled the evaluation of the select metabolites monitored in this study [31,32]. Samples of CFE were injected into an Alliance HPLC equipped with a C18 reverse-phase column (Synergi Hydro-RP; 4  $\mu\text{m}$ ; 250  $\times$  4.6 mm, Phenomenex) operating at a flow rate of 0.7 mL/min at ambient temperature. A mobile phase consisting of 20 mM  $\text{KH}_2\text{PO}_4$  (pH 2.9) was utilized to separate organic acids, which were identified using a Waters dual absorbance detector at 210 nm. Nucleotides were monitored at 254 nm. Select metabolites were detected with the aid of known standards, and peaks were quantified with the Empower software (Waters Corporation) [31]. Enzymes such as NDPK and AK were monitored by incubating the soluble cell-free extract (CFE) and/or the excised activity bands with the appropriate substrates. To detect NDPK activity, 2 mM GDP and 2 mM ATP were the substrates while for AK, 2 mM ATP and 2 mM AMP were utilized. The consumption and formation of nucleotides were gauged by HPLC following a 30 min incubation period. The sample (100  $\mu\text{L}$ ) was collected and diluted ten-fold with Milli-Q water and injected immediately after the reactions in order to minimize substrate and product degradation. These experiments helped compare the amounts of GTP and ATP generated in the control and stressed situations respectively. Internal standards were included to ensure equal volume loading.

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