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The role of formate in combatting oxidative stress

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

Keywords Formate metabolism · *Pseudomonas fluorescens* · Ketoacids · Glyoxylate · Formate dehydrogenase · Fumarate reductase- formate dependent · Isocitrate lyase

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Introduction

As oxidative stress is part of aerobic life, most organisms have evolved intricate mechanisms to circumvent this challenge. These strategies include the utilization of enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase which aid in the lowering of oxidative tension during aerobic respiration (Brioukhanov et al. 2006). Recently the significance of ketoacids in combatting reactive oxygen species (ROS) has emerged. The moieties such as alpha ketoglutarate (AKG), oxaloacetate (OAA), pyruvate and glyoxylate scavenge ROS with the concomitant formation of their respective carboxylic acid (Alhasawi et al. 2015a; Lemire and Appanna 2011; Mailloux et al. 2008; Singh et al. 2008; Li et al. 2009; Thomas et al. 2015). When glyoxylate is utilized as an ROS scavenger, formate is one of the critical by-products formed (Alhasawi et al. 2015b; Yokota et al. 1983; Yokota et al. 1985). In numerous bacteria, plants and animals formate is an important metabolite

involved in energy metabolism (Hourton-Cabassa et al. 1998; Leonharsberger et al. 2002). Its favourable redox potential enables this monocarboxylic acid to be oxidised not only through the aerobic respiratory pathways but to serve as an electron donor for the reduction of key metabolites such as fumarate, nitrate and nitrite (Bagramyan et al. 2000; Jormakka et al. 2002; Shinagawa et al. 2008; Su and Puls 2004).

Formate may undergo different fates in these bacteria depending on the physiological conditions (Leonharsberger et al. 2002). It may be secreted, oxidised aerobically, used as a reductant or converted into CO_2 and H_2 via the enzyme formate hydrogen lyase (Bagramyan and Trchounian 2003; Yoshida et al. 2005). A key enzyme in the metabolism of formate is formate dehydrogenase (FDH). In some bacteria this enzyme predominantly utilizes nitrate as an electron acceptor (Uchimura et al. 2002). FDH plays a pivotal role in respiration as well as in the maintenance of a reducing environment (Jormakka et al. 2002). These enzymes have been reported with differing cofactor requirements, electron acceptors, substrates and cellular locations (Hourton-Cabassa et al. 1998). The FDHs NAD-dependent which have been extensively studied in both bacteria, yeast and plants, have been widely utilized in industry for NADH regeneration (Alekseeva et al. 2011; Hoelsch et al. 2013; Suzuki et al. 1998).The occurrence of FDH NADP-dependent has also been reported (Yammamoto et al. 1983; Gul-Karaguler et al. 2001). FDH synthesis has been shown to increase strongly under conditions of stress including abrupt changes in temperature, irradiation with UV light, hypoxia and chemical agents and may contribute in maintaining NADPH homeostasis (Andreadeli et al. 2009; Andreadeli et al. 2009; Hoelsch et al. 2013; Hourton-Cabassa et al. 1998; Jormakka et al. 2003).

The role of this monocarboxylic acid in providing the reducing power to the pivotal ribonucleotide reductase has been shown (Stubbe et al. 2003). This enzyme participates in the synthesis of deoxyribonucleotides with the concomitant formation of CO_2. Deoxyribonucleotides are critical in the cellular replication and their formation usually necessitates the utilization of NADPH. As part of our study to unravel the significance of metabolic pathways in antioxidative defence, we have evaluated the role of formate in the adaptation of the nutritionally-versatile microbe *Pseudomonas fluorescens* to oxidative stress. Here we report that the presence of the enhanced level of this mono- carbon carboxylic acid is an important source of reducing power in the H_2O_2-challenged cells. It participates in the synthesis of NADPH and contributes to the reduction of fumarate to succinate, biological reactions that ensure the survival of the microbe in an oxidative milieu. The importance of formate as a substitute for NADH in environments where the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OP) are ineffective is also discussed.

Methods

Growth

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

Regulation and whole cell experiments

In the regulation experiments, to assess the adaptive and reversible nature of the shifts in metabolism, cells were harvested at stationary phase of growth. Control

cells (10 mg protein equivalent) were incubated for 8 h in 50 mL media containing 500 μM H_2O_2 whereas the H_2O_2-stressed cells were incubated for 8 h in 50 mL control media as described in (Alhasawi et al. 2014). The cell-free extracts and the spent fluid were subsequently analyzed for metabolites and enzymatic activities.

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

Cell fractionation

Following the isolation of the bacteria at 4 °C for 10 min at 10,000 g with the aid of a Sorvall Legend RT Centrifuge, cells were washed with 0.85 % NaCl and respun before being resuspended in 500 μL cell storage buffer (CSB) consisting of 50 mM Tris–HCl, 5 mM $MgCl_2$ and 1 mM fluoride (PMSF). Sonication was utilized to lyse the cells (unbroken cells were removed by centrifugation at 10,000 g). These were centrifuged at 180,000 g for 3 h at 4 °C yielding a soluble cell-free (CFE) and a membrane fraction. The membrane fraction was suspended in 500 μL of CSB. The Bradford assay was utilized to determine protein content with serum bovine albumin as the standard. Equal protein concentrations were utilized in all experiments.

Enzymatic studies

BN-PAGE was executed as per the protocol described in, Auger et al. (2015); Mailloux et al. (2008) and Schagger and von Jagow (1991). For these assays, a 4–16 % gradient gel was prepared and the protein (4 μg/μL) was prepared in blue native buffer (400 mM 6-amino hexanoic acid, 50 mM Bis–Tris [pH 7.0]). To solubilize membrane bound proteins in order to ensure optimal protein separation, a final concentration of 1 % dodecyl-β-maltoside was added to the membrane fractions. Protein samples were loaded into each well of the native gel (10–60 μg) and electrophoresed at 4 °C under native conditions at 80 V and 15 mA for proper stacking followed by 150 V and 25 mA in the resolving gel for the migration of the protein until it travelled half-way through the gel. At the halfway point, blue cathode buffer (50 mM Tricine, 15 mM Bis–Tris, 0.02 % w/v Coomassie G-250, pH 7 at 4 °C) was changed to a colorless cathode buffer (50 mM Tricine, 15 mM Bis–Tris, pH 7 at 4 °C) to provide improved detection of the protein bands and thence electrophoresis was performed at 300 V and 25 mA. For 15 min following the electrophoresis, the gel was incubated in reaction buffer (25 mM Tris–HCl, 5 mM $MgCl_2$ [pH 7.4]), after which, the in-gel activity assay was performed by using a reaction mixture containing equilibrium buffer, 5 mM substrate, 0.5 mM cofactors, 0.2 mg/mL phenazine methosulfate (PMS) or dichloroindophenol (DCIP), and 0.5 μg/mL iodonitrotetrazolium (INT) in a total volume of 3 mL. For FDH-NADP, this consisted of 5 mM formate and 0.5 mM NADP whereas the FDH-NAD utilized 0.5 mM NAD instead. To confirm the presence of these enzymes the activity bands were cut and incubated in 1 mL reaction mixtures containing the corresponding substrates bicarbonate and NADPH and NADH respectively while monitoring the formation of formate via HPLCs. Fumarate reductase was detected using 5 mM fumarate and 0.5 mM formate. Confirmation was obtained by incubating the excised bands in a mixture containing fumarate (5 mM) and formate (0.5 mM) and monitoring for succinate production. Isocitrate lyase, Complex I and NADH were monitored as described in (Auger et al. 2015). Destaining solution (40 % methanol and 10 % glacial acetic acid) was used to stop the reactions where appropriate. Coomassie staining was used to ensure equal protein loading. The specificity in detections was further confirmed by performing in-gel reactions in the absence of a substrate or by the addition of the inhibitor sodium azide (5 mM). Densitometry was performed using imageJ for windows.

Spectrophotometric data for NAD-dependent isocitrate dehydrogenase (ICDH-NAD) was obtained by incubating 1 mg (protein equivalent) of membrane fraction from control and H_2O_2-treated cells with 2 mM isocitrate and 0.5 mM NAD for 1 min. For NADP-dependent ICDH, NADP was used instead of NAD. A similar reaction was used for malic enzyme (ME) except NAD was replaced with NADP and

malate was utilized instead of ICDH. NADH and NADPH production were monitored at 340 nm over the course of a minute. Negative controls were performed without the substrates or cofactors.

Metabolite analysis

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

Statistical analysis

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

Results and discussion

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

As formate was a key metabolite in the stressed cells, it was important to evaluate how this moiety was contributing to the anti-oxidative defence strategy of the microbe. There was a marked increase in the activity of FDH-NADP in the stressed cells (Fig. 2a).[2] In an effort to ascertain if this enzyme was being expressed as a consequence of oxidative stress, control cells were exposed to H_2O_2 medium while H_2O_2 challenged cells were incubated in a control medium. A marked reduction of FDH-NADP activity band was observed in the latter while in the former situation the activity band corresponding to the dehydrogenase was enhanced (Fig. 2b). FDH-NADP was readily inhibited by sodium azide (Fig. 2c). This enzyme may help contribute to the NADPH budget which is critical in combatting oxidative stress, as both malic enzyme and ICDH-NADP that are known to synthesize this reducing agent were also increased (Table 1) (Beriault et al. 2005, 2007; Ying 2008). The stressed cells were

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

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

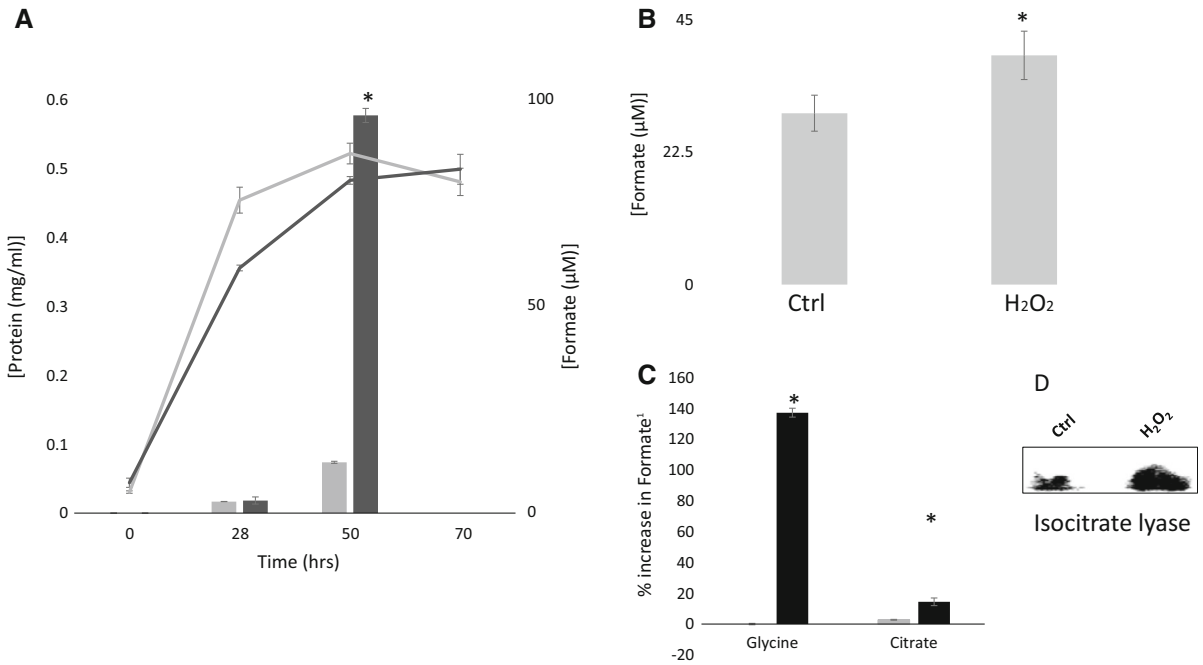

Fig. 1 Formate production in *P. fluorescens* **a** Bacterial cell growth as measured by the Bradford assay (protein (mg/ml of culture) and formate production (monitored (by HPLC) in the growth media at various time intervals (*filled square* control, *filled square* 500 μM H_2O_2). **b** Formate levels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

also characterized by an increase in FDH-NAD (Fig. 2d) which was confirmed by excision of the activity band and incubation in reaction mixture containing NADH and bicarbonate to monitor formate production (Fig. 2e). Furthermore this trend was reversed in the regulation experiment as observed (Fig. 2f). However, the presence of H_2O_2 resulted in the diminution of the activities of TCA cycle enzymes such as ICDH-NAD (Table 1). Also, Complex I was marked diminished in the stressed cultures (Fig. 2g) while the activity band indicative of NADH oxidase was barely evident in the control cells (Fig. 2h).

Fumarate reductase (FRD) mediates the conversion of fumarate into succinate with concomitant oxidation of NADH (Appanna et al. 2014). Although this enzyme was present, it readily utilized formate as the reducing cofactor (Fig. 3a).[3] Formate is known to provide electrons with the liberation of CO_2 (Zaunmuller et al. 2006). There was a drastic increase in formate dependent FRD in the stressed cells compared to the control cells where the activity band was only slightly visible (Fig. 3a). Regulation experiments confirmed the reversible nature of this enzyme (Fig. 3b). Furthermore, the formate dependent FRD was distinguished from the NAD-dependent FRD which also showed a marked increase in stress cells (Fig. 3c). Incubation of the excised activity band of formate dependent FRD in fumarate and formate yielded succinate (Fig. 3d). The membrane fraction from the stressed cells incubated in reaction mixture containing fumarate, formate and ADP for 30 min generated more ATP and succinate compared to the control cells (Fig. 3e).

The data in this report point to the ability of formate to act as an important reducing factor in *P. fluorescens* exposed to oxidative stress. This carboxylic acid that is referred to as reduced carbon dioxide is known to

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

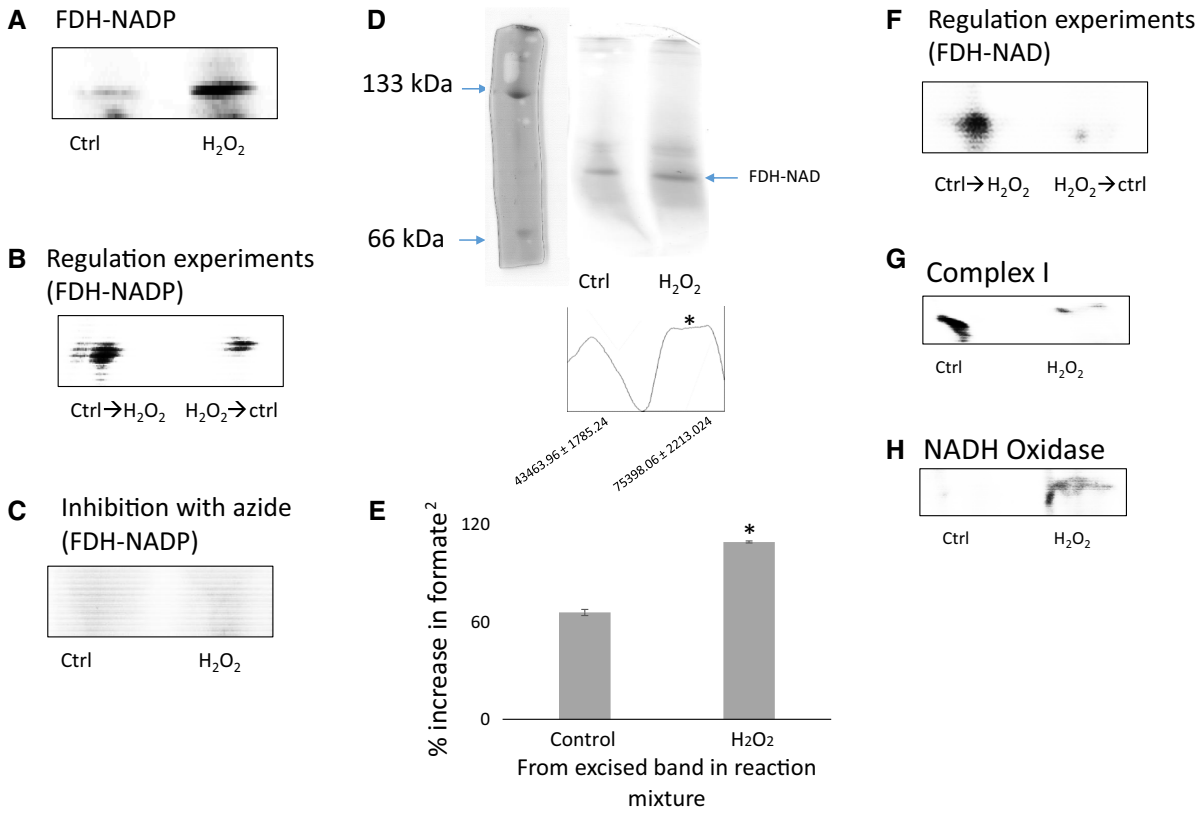

Fig. 2 NADH homeostasis under oxidative stress. **a** In-gel activity of FDH-NADP. **b** In-gel activity of FDH-NADP following regulation experiments. **c** In-gel activity of FDH-NADP using azide inhibitor to ensure specificity of enzyme. **d** In-gel activity of NAD-dependent formate dehydrogenase. **e** Percent increase in formate production from incubation of excised activity bands of FDH-NAD following BN-PAGE, for 30 min in reaction mixture containing 0.5 mM NADH and 5 mM bicarbonate. **f** In-gel activity of FDH-NAD following regulation experiments. **g** In-gel activity of complex I. **h** In-gel activity of NADH oxidase between control and stress cells. Gels are representative of at least 3 independent trials. *Asterisks* represents statistical significance in comparison to control; n = 3, $p < 0.05$, mean ± S.D

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

Enzyme name	Control	H_2O_2
ME	2.42 ± .59	4.29 ± 1.47
ICDH-NADP	1.38 ± .88	2.63 ± .29
ICDH-NAD	1.13 ± .27	.57 ± .09

µmol NAD(P)H produced min-1 mg protein-1 as monitored at 340 nm (n = 3 ± standard deviation)

ME malic enzyme, *ICDH-NAD* NAD dependent isocitrate dehydrogenase, *ICDH-NADP* NADP dependent isocitrate dehydrogenase

provide the reducing fuel in a variety of biochemical reactions in lieu of NADH and NADPH. Reduction of ribonucleotide, nitrite and cytochrome C has been shown to be mediated by formate (Stubbe et al. 2003). In this study, formate contributes to the anti-oxidative defense strategy by supplying NADPH. Although a variety of mechanisms are deployed by microorganisms including the synthesis of exopolysaccharides (Appanna and Preston 1987) to quell oxidative tension, there is a dearth of information on such a role for this carboxylic acid. This metabolite aided by the enzymes ME and ICDH-NADP, may allow *P. fluorescens* to battle the oxidative challenge posed by H_2O_2. Indeed, various organisms are shown to evoke intricate NADPH-generating pathways to modulate their NADPH budget to combat oxidative stress (Alhasawi et al. 2014; Chenier et al. 2008; Mailloux et al. 2009). The ability of formate to reduce fumarate to succinate with the aid of FRD may prove an added

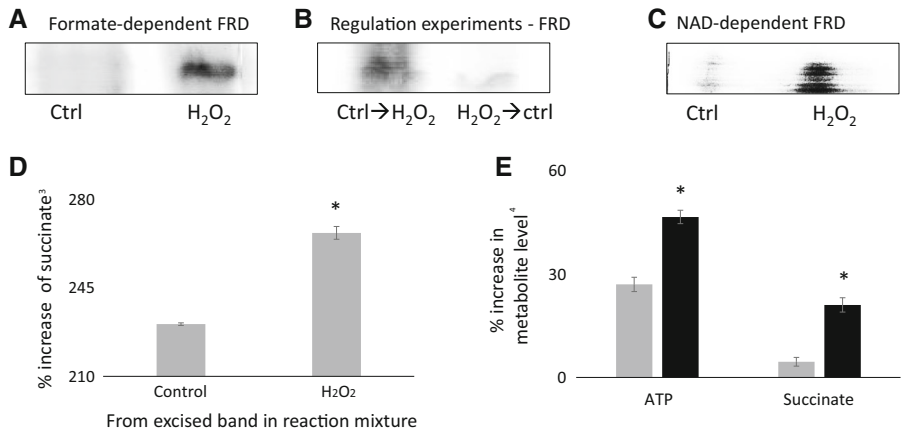

Fig. 3 Formate-dependent succinate production. **a** In-gel activity of formate-dependent fumarate reductase (FRD). **b** In-gel activity of formate-dependent fumarate reductase following regulation experiments. **c** In-gel activity of NAD-dependent fumarate reductase. **d** Percent increase in succinate production from excised bands following BN-PAGE incubated for 30 min in reaction mixture containing 5 mM fumarate and 0.5 mM formate. **e** Percent increase in metabolites (ATP and succinate) from an HPLC reaction containing *Pseudomonas fluorescens* membrane fraction incubated in reaction buffer with 2 mM fumarate, 0.5 mM formate and 0.5 mM ADP for 30 min (*filled square* control, *filled square* 500 μM H_2O_2). Gels are representative of at least 3 independent trials. *Asterisks* represents statistical significance in comparison to control; $n = 3$, $p < 0.05$, mean ± S.D. (0–100 %)

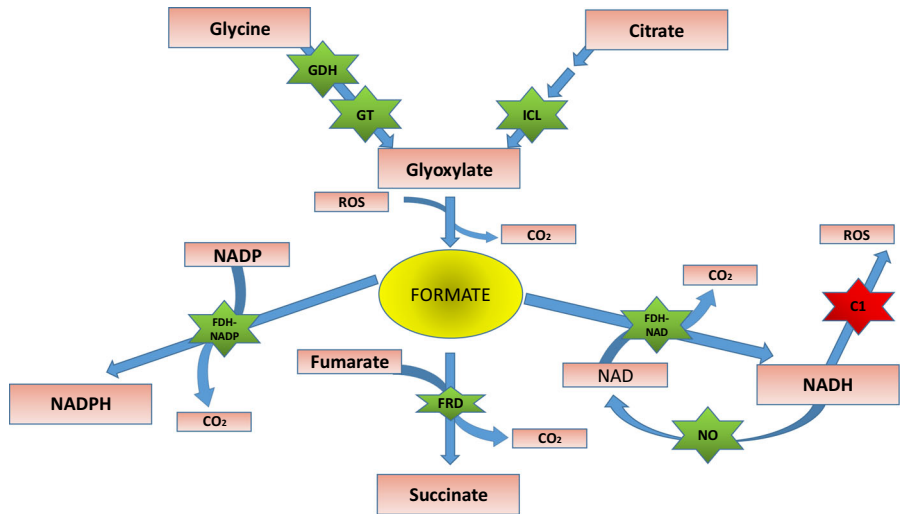

Fig. 4 Schematic demonstrating the metabolic shift involving the role of formate in *P. fluorescens* in combatting oxidative stress (*GDH* glycine dehydrogenase, *GT* glycine transaminase, *ICL* isocitrate lyase, *FDH-NADP* NADP-dependent formate dehydrogenase, *FRD* fumarate reductase-formate dependent, *FDH-NAD* NAD-dependent formate dehydrogenase, *NO* NADH oxidase, *C1* Complex I). = increase in enzyme activity = decrease in enzyme activity

benefit to this microbe as the production of NADH is markedly diminished under oxidative tension (Mailloux et al. 2011). The TCA cycle, a key generator of the catabolic reducing agent, is severely impeded (Bignucolo et al. 2013; Mailloux et al. 2007). Additionally, oxidative phosphorylation is downregulated as revealed by the diminished activity of Complex I, a situation that may impede the generation of NAD. To rectify such an occurrence, the microbe invokes the participation of NADH oxidase, an enzyme whose activity is known to be increased during environmental stress (Chenier et al. 2008). Although FDH-NAD

may help alleviate the diminished NADH production in the H_2O_2 medium, the utilization of formate in NADH-requiring processes like in the reduction of fumarate to succinate will be an added benefit during oxidative stress. Hence, reactions necessitating NADH may switch to formate as a reducing factor. The enhanced synthesis of glyoxylate fuelled by the increased activity of ICL may argue for such a possibility. It is quite likely that *P. fluorescens* may have adopted this strategy. Formate, a by-product of the detoxification of ROS by glyoxylate, may have aptly been utilized by this microbe as a potent reductive power.

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

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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[4] These values are relative to the respective metabolite levels in the reaction mixtures at time 0.

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