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The overexpression of NADPH-producing enzymes counters the oxidative stress evoked by gallium, an iron mimetic

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Abstract

Gallium (Ga), an iron (Fe) mimetic promoted an oxidative environment and elicited an antioxidative response in *Pseudomonas fluorescens*. Ga-stressed *P. fluorescens* was characterized by higher amounts of oxidized lipids and proteins compared to control cells. The oxidative environment provoked by Ga was nullified by increased synthesis of NADPH. The activity and expression glucose 6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase-NADP (ICDH) were stimulated in Ga-cultures. The induction of isoenzymes of these dehydrogenases was also evident in the Ga-stressed cells. Although superoxide dismutase (SOD) activity was significantly enhanced in Ga-stressed cultures, catalase activity experienced a marked diminution. Fe metabolism appeared to be severely impeded by Ga toxicity. This is the first demonstration of the oxidative stress evoked by Ga to be neutralized by a reductive environment generated via the overexpression of NADPH-producing enzymes.

Introduction

Metals are an important component of all living organisms as they participate in a wide array of biochemical reactions essential for life. While Ca is essential as a signaling element, Mg is critical in all reactions necessitating ATP (Williams 1999). The redox metals like Fe and Cu due to their ability of accepting and giving electrons play a pivotal role in oxidative phosphorylation (Menzies *et al.* 2002). However, there are some metals that have no known biological functions and are usually toxic. Al, Cd and Hg belong to this category of metals. They exert their toxicity by substituting for essential metals and binding to functional proteins (Bruins *et al.* 2000). Al interacts with Fe proteins and helps increase the labile iron pool, a situation that has been shown to promote the production of reactive oxygen species (ROS) (Harris *et al.* 2003; Ozgova *et al.* 2003; Middaugh *et al.* 2005).

As ROS are generated due to normal cellular metabolism, most organisms have evolved an intricate system to combat the toxicity associated with these moieties. Catalase, SOD and glutathione are some of the biomolecules involved in oxidative defense (Jarrett & Boulton 2005). If the concentration of intracellular ROS is not properly controlled, cellular damage may lead to cellular death. In fact, numerous diseases are known to be initiated by abnormal ROS formation (Manfredi & Xu 2005; Okada *et al.* 2005). A dysfunctional Fe metabolism has been shown to result in unregulated ROS production (Huang 2003). For instance, the amyloid β-peptide, a marker for Alhzeimer's Disease (AD), has been shown to generate H_2O_2 in the presence of free Fe (Rottkamp *et al.* 2001). As

part of our study to elucidate the molecular details associated with the toxicity and the subsequent adaptation evoked by metal stress, we have examined the impact of Ga on the microbe, *Pseudomonas fluorescens*. Ga provides a unique model system to evaluate the biochemical changes associated with the abnormal metabolism of the essential metal Fe. As Ga mimics Fe and does not participate in the transfer of electrons, it is known to render Fe–proteins biologically ineffective. This feature of Ga has been exploited to target aggressively dividing cells like cancer cells. Since these cells have an insatiable demand for Fe, Ga is utilized to visualize and kill these cells (Chitambar 2003). In the present report, we have investigated how the toxic influence of Ga is manifested and the mechanisms invoked to counter this toxicity have also been elucidated. Here, we show that Ga promotes the formation of ROS, perturbs Fe metabolism and evokes the increased synthesis of NADPH via the overexpression of the enzymes G6PDH and ICDH–NADP. The enhanced genesis of NADPH appears to be a key component of the antioxidative strategy elaborated by this organism to survive Ga stress.

Materials and methods

Bacterial culture and cell free extracts

Pseudomonas fluorescens ATCC 13525 were maintained on 2% agar and grown in a mineral medium consisting of Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4$ (0.2 g) and citric acid (4.0 g) per liter of deionized distilled water. Trace elements ($FeCl_3 \cdot 6H_2O$ (2 μM); $MgCl_2 \cdot 4H_2O$ (1 μM); $Zn(NO_3)_2 \cdot 6H_2O$ (0.05 μM); $CaCl_2$ (1 μM); $CoSO_4 \cdot 7H_2O$ (0.25 μM) $CuCl_2 \cdot 2H_2O$ (0.1 μM); $NaMoO_4 \cdot 2H_2O$ (0.1 μM) were also added as described in (Anderson *et al.* 1992). In the Ga-stressed medium, citric acid was complexed with Ga^{3+} in a ratio of 19 mM citrate to 1 mM Ga $(NO_3)_3$. The pH was adjusted to 6.8 with dilute NaOH and 200 ml amounts of media were dispensed in 500 ml Erlenmeyer flasks. Inoculations were made with 1 ml of stationary phase cells grown in a medium unamended with the test-metal and aerated on a gyratory waterbath shaker, model 76 (New Brunswick Scientific) at 26 °C.

Cells were collected by centrifugation (10,000 $\times$ *g* for 10 min at 4 °C) and washed twice with 0.85% (w/v) NaCl and once with cell storage buffer (100 mM Tris–HCl (pH 7.3), 1 mM DTT, and 1 mM PMSF). Cells were ultrasonically disrupted in the cell storage buffer with the aid of a Brunswick sonicator, power level 4 for 15 s at 4 intervals. The supernatant fraction of the CFE was collected and centrifuged at 180,000 $\times$ *g* for 60 min at 4 °C to yield membrane and soluble components. The soluble fraction was further centrifuged at 180,000 $\times$ *g* for 2 h to afford a membrane-free system. Fractions were kept on ice in the refrigerator or frozen at -20 °C for storage for a maximum of 4 weeks. Protein concentrations were measured using the Bradford method and bovine serum albumin (BSA) was the standard (Bradford 1976).

TBARS and protein carbonyl analyses

CFE from Ga-stressed and control cells were probed for the presence of thiobarbituric acid reactive substances (TBARS) at various growth intervals as described in (Buege & Aust 1978). Briefly, 2 mg of protein equivalent of inner membrane were heated with 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid and 0.25 N HCl to a final volume of 1.0 ml for a period of 15 min. The samples were centrifuged at 10,000 $\times$ *g* for 10 min and the absorbance of the supernatant fraction was measured at 532 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

The total protein carbonyl content was analyzed according to the method described in (Vendemiale *et al.* 2001) with the following modifications. Control and Ga-stressed cultures were harvested at stationary phase of growth. Soluble CFE (1 mg protein equivalent) was allowed to react with 2% (w/v) 2,4-dinitrophenylhydrazine (DNPH). Following a 60 min incubation, 200 μl of 50% (w/v) TCA was added to each sample. The resulting precipitate was removed by centrifugation at 23,500 $\times$ *g* for 10 min. The supernatant fraction was discarded and the pelleted protein was washed with 10% (w/v) TCA and centrifuged again at 23,500 $\times$ *g* for 10 min. After two more washes in 10% (w/v) TCA, the protein pellet was washed thrice with a solution of ethylacetate:ethanol (1:1). The protein pellet was then dissolved in 1 ml of 6 M guanidine and the

absorbance at 370 nm (ε = 21.5 M^{-1} cm^{-1}) was recorded.

Measurement of ROS

The CFE (3 mg protein equivalent) from control cultures were incubated at room temperature in a reaction mixture containing 25 mM Tris–HCl (pH 7.3), 5 mM $MgCl_2$. Peroxide was measured by adding 4 units of peroxidase, 10 mM p-anisidine, and citrate (5 mM) or Ga-citrate (5 mM) to a final volume of 1 ml. Following a 30 min incubation the peroxide produced was quantified colorimetrically at 458 nm (ε = 1.73 M^{-1} cm^{-1}) (Munoz *et al.* 1997). The CFE (3 mg protein equivalent) from control cultures were incubated at room temperature in a reaction mixture containing 25 mM Tris–HCl (pH 7.3), 5 mM MgCl. Superoxide was measured by adding 0.12 mM INT, and citrate (5 mM) or Ga-citrate (5 mM) to a final volume of 1 ml. Following 1–5 h incubation the superoxide produced was quantified colorimetrically at 485 nm (ε = 11 M^{-1} cm^{-1}) (Poinas *et al.* 2002). As we were unable to detect $O_2^{\bullet-}$ in the presence of Ga, the influence of Ga on the production of $O_2^{\bullet-}$ was studied by electron paramagnetic resonance (EPR) spectroscopy. To obtain a spectrum for $O_2^{\bullet-}$, xanthine oxidase was utilized. Reactions were initiated by the addition of xanthine oxidase at 0.8 U ml^{-1} in incubation mixtures containing xanthine (1 mM), DMPO (50 mM), EDTA (0.2 mM) and phosphate buffer (0.1M, pH 7.4) in order to generate $O_2^{\bullet-}$. The spectra of this ROS were also recorded in the presence of citrate, Ga-citrate and Al-citrate, respectively in the reaction mixture. A Varian E-109 EPR spectrometer operating at *x*-band was utilized. The perturbation in Fe metabolism was also followed spectrophotometrically by examining the peak of the 395–420 nm region, that has been shown to be a fingerprint of Fe–S cluster (Middaugh *et al.* 2005). Soluble CFE obtained from control and Ga-stressed cells at the same growth phase were analyzed (note: same amounts of proteins were utilized).

Monitoring of enzymatic activities

Catalase activity was determined spectrophotometrically using p-anisidine at 458 nm (Igamberdiev *et al.* 1995). One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min per mg protein in a 15 mM H_2O_2 solution at pH 7.3 at 26 °C. The enzyme reaction was carried out in 1 ml of 25 mM Tris–HCl buffer with 5 mM $MgCl_2$ and soluble CFE (200 μg protein equivalents) and 10 mM p-anisidine. SOD activity was determined as described in (Beyer & Fridovich 1987) with the following modifications. Activity was monitored spectrophotometrically with the aid of INT (ε = 11 M^{-1} cm^{-1}) at 485 nm. The enzyme reaction was carried out in 1 ml of 25 mM Tris–HCl buffer with 5 mM $MgCl_2$ and 200 μg soluble CFE protein equivalent and 5 mM menadione, a superoxide generating compound, and 60 μg ml^{-1} INT. The activity of Ga-stressed cells grown to 55 h was taken as 100%.

ICDH-NADP was analyzed in a 1 ml reaction mixture containing 25 mM Tris–HCl (pH 7.3), 5 mM $MgCl_2$, 2 mM isocitrate, 4 mM malonate, 0.2 mM $NADP^+$. Enzyme activity was monitored by following the formation of NADPH at 340 nm (ε = 6270 M^{-1} cm^{-1}) and by quantifying α-ketoglutarate formation colorimetrically with DNPH (ε = 16,000 M^{-1} cm^{-1}) (Romanov *et al.* 1999). α-ketoglutarate was used as the standard. One unit of ICDH is defined as the amount that produces 1 μmol of 2,4-dinitrophenylhhydrazone or produces 1 nmol NADPH in 1 min per mg of protein in a solution of 0.2 mM $NADP^+$. G6PDH activity was determined spectrophotometrically by the method described by Wynn *et al.* (1997) with the following modifications. G6PDH was analyzed in a 1 ml reaction mixture containing 25 mM Tris–HCl (pH 7.3), 5 mM $MgCl_2$, 2 mM isocitrate, 4 mM malonate, 0.2 mM $NADP^+$. Enzyme activity was monitored by following the formation of NADPH at 340 nm (ε = 6270 M^{-1} cm^{-1}). Malic enzyme (ME) was monitored by analyzing the production of NADPH at 340 nm in CFE incubated with malate and $NADP^+$. 6-Phosphogluconate dehydrogenase (6-PGDH) was quantitated by monitoring NADH at 340 nm in the presence of 6-phosphogluconate as the substrate (Singh *et al.* 2005).

BN-PAGE, SDS PAGE, Western Blot analyses and Enzyme activity staining

Blue Native Polyacrylamide gel electrophoresis (BN-PAGE) was performed according to the method of Schägger & von Jagow (1991). BioRad

MiniProtean^{TM} 2 system was utilized and gels with a linear gradient (10–16%) were selected to separate the proteins. Soluble protein samples were prepared in BN buffer (50 mM BisTris, 500 mM 6-aminohexanoic acid, pH 7.0), and 20–80 μg protein per lane was loaded to each well. Gels were electrophoresed under BN conditions; 80 V was used for the stacking gel and was increased to 200 V when the proteins reached the separating gel. The blue cathode buffer (50 mM Tricine, 15 mM Bis-Tris, 0.02% (w/v) Coomassie blue G-250, pH 7.0 at 4 °C) was changed to the colorless cathode buffer (50 mM Tricine, 15 mM Bis-Tris, pH 7.0 at 4 °C) once the running front reached halfway through the separating gel. Electrophoresis was stopped before the running front was out of the gel. The gels were subsequently placed in an equilibration buffer (25 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$) for 15 min.

ICDH activity was visualized by the formation of formazan in the equilibration buffer which consisted of 1 mM isocitrate, 0.1 mM $NADP^+$, 0.4 mg ml^{-1} iodonitrotetrazolium (INT), and 0.2 mg ml^{-1} phenazine methosulfate (PMS). G6PDH was detected with the same technique except glucose 6-phosphate (5 mM) was utilized as the substrate (Beriault *et al.* 2005). Catalase activity was visualized with the aid of p-anisidine. Following a 5 min incubation in the equilibration buffer, the gels were transferred to equilibration buffer containing 10 mM p-anisidine and 35 mM H_2O_2. For the visualization of SOD, the gels were placed in the equilibration buffer consisting of 0.5 mg ml^{-1} INT and 15 mM menadione. Activity was evident as an achromatic band against a deeply colored gel (Caldwell & Hassan 2002).

Results

Ga toxicity and ROS production

P. fluorescens has previously been shown to tolerate 1 mM of Ga. After a lag phase of 30 h, the microbe grew as fast as the control cultures (al-Aoukaty *et al.* 1992). However, the exact biochemical mechanism that contributes to this adaptation to Ga is not well established. As Ga has been suggested to be a pro-oxidant (Exley 2004), the amount of oxidized lipids and proteins were monitored. When compared to control cells, Ga-stressed cells showed increased levels of oxidized lipids. Figure 1 depicts an approximate 2-fold increase in lipid peroxidation at late log phase and early stationary phase. In addition protein carbonyl levels also increased in cells exposed to Ga-citrate. At the same phase of growth i.e. 20 h for the control culture and 65 h for the stressed cultures, there was at least 60% more of carbonyl per mg of protein in the Ga-stressed cells (data not shown).

The pro-oxidant activities of Ga were investigated in CFE from *P. fluorescens* cultures. Following the incubation of CFE with citrate or Ga-citrate, H_2O_2 levels were measured. Figure 2 clearly indicates the presence of H_2O_2 in the CFE exposed to Ga-citrate while the CFE treated with citric acid did not have any detectable H_2O_2. The CFE from Ga-stressed cultures were exposed to both citrate and Ga-citrate, respectively and $O_2^{\bullet-}$ was monitored. When citrate was the substrate, this moiety was observed. In the presence of Ga-citrate, $O_2^{\bullet-}$ was not discerned. Thus, it appeared that Ga interfered with $O_2^{\bullet-}$ production and/or modified the nature of this ROS as it was not detectable by the colorimetric assay. To further probe this finding, EPR studies were performed. The addition of DMPO to a phosphate buffer solution containing xanthine oxidase, xanthine and EDTA resulted in the formation of the hydroxyl radical adduct of DMPO with a characteristic spectrum centered at $g = 2.01$. The addition of citrate to this reaction mixture did not appear to have any noticeable effect. However, when Ga-citrate was included these peaks were absent. In the presence of Al-citrate the peaks attributable to interaction of $O_2^{\bullet-}$ and DMPO were less intense (Figure 3). Thus, it appeared that Ga was quenching the $O_2^{\bullet-}$ signal.

Catalase, SOD and Fe homeostasis

The ROS stress imposed by the presence of Ga provided impetus to investigate the enzymes involved in ROS detoxification. Catalase, an important enzyme involved in the degradation of H_2O_2 was monitored with the aid of BN-PAGE. Figure 4 shows catalase activity in *P. fluorescens* exposed to citrate and Ga-citrate at various growth intervals. The Ga-stressed cells had a maximal decrease in activity at 65 and 70 h. The

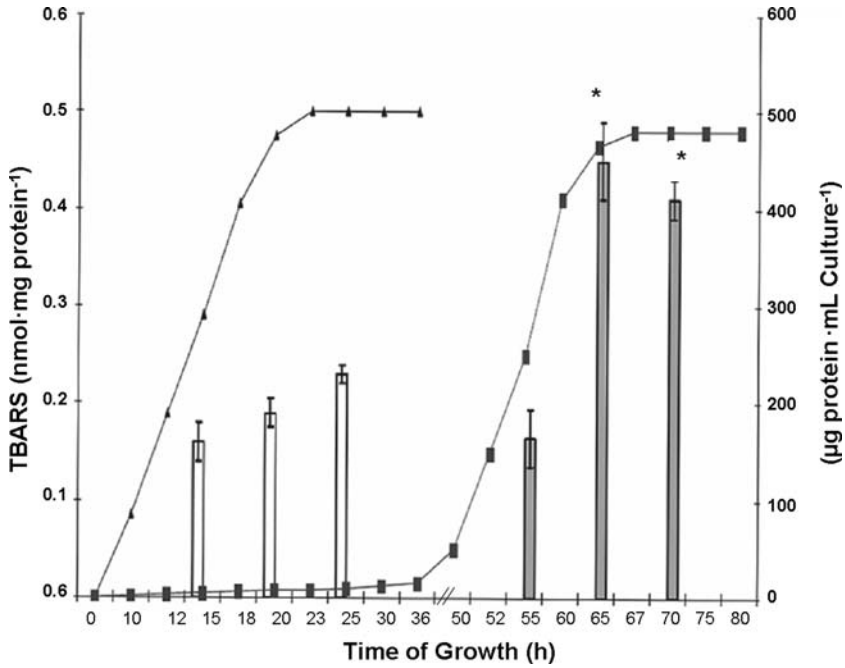


Figure 1. Oxidized lipids in control (clear bars) and Ga-stressed (dark bars) *P. fluorescens* at various growth intervals (▲ = control; ■ = Ga-stressed cells) ($n = 3$) (2 mg of protein equivalent of inner membranes were utilized.Values are means ± S.D. TBARS values differ significantly from control (*) $P \leq 0.05$.

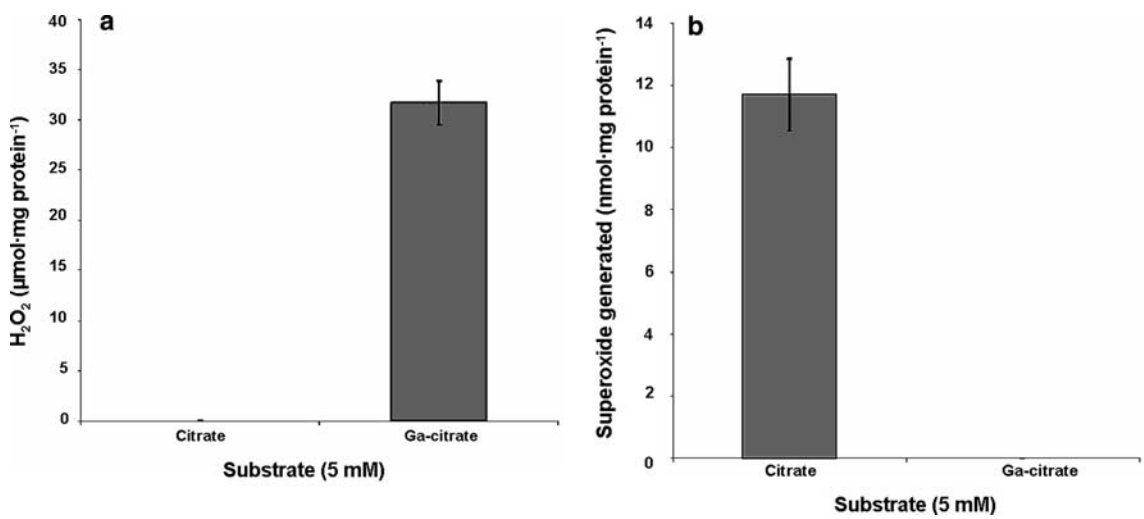


Figure 2. (A) H_2O_2 production by the membrane fractions from *P. fluorescens* ($n = 3$). Note: no H_2O_2 was detected when citrate was the substrate. (Ga-stressed cells were utilized). (B) $O_2^{\cdot -}$ production by the membrane fractions from *P. fluorescens* ($n = 3$) (Ga-stressed cells were used).

electrophoretic data corresponded with the spectrophotometric results. When exposed to Ga-stress, there was a marked decrease in catalase activity. The possibility that the interference of Ga with Fe might be a cause for decreased catalase activity was investigated. The absorption peak in the 395–420 nm, that is indicative of an Fe–S cluster (Soum *et al.* 2003; Berndt *et al.* 2004; Middaugh *et al.* 2005), was markedly diminished in the Ga-stressed cells (Figure 5).

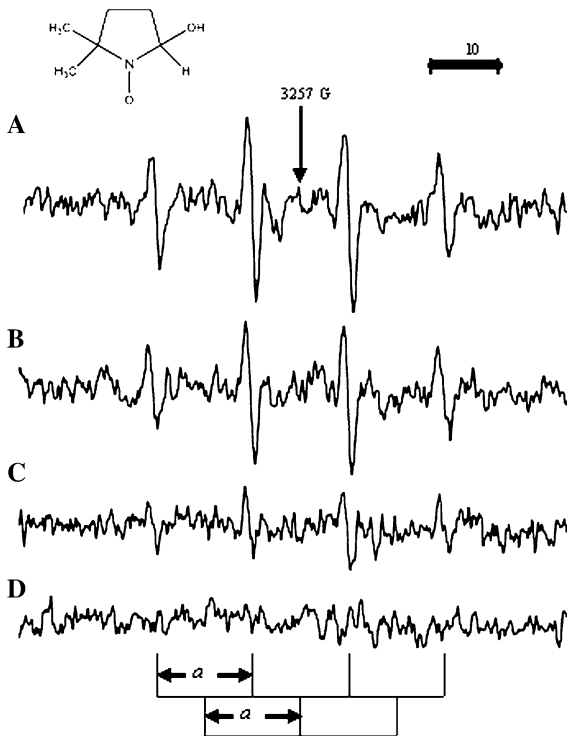


Figure 3. Superoxide quenching activity of Ga-citrate. Spectra were recorded after incubation of: (A) xanthine oxidase (0.8 U ml^{-1}) with xanthine (1 mM), EDTA (0.2 mM), and DMPO (50 mM) in phosphate buffer (0.1 M, pH 7.4) in a total volume of 20 μl; (B) as A plus citrate (5 mM); (C) as A plus Al-citrate (1:1 ratio of 5 mM); (D) as A plus Ga-citrate (1:1 ratio of 5 mM) in phosphate buffer (0.1 M, pH 7.4). Samples were analyzed using the following instrumental conditions: microwave power 1.5 mW, time constant 0.250 ms and gain 1.0×10^5.

Despite the inability to detect superoxide species in the CFE of cells treated with Ga-citrate, the activity of SOD was examined. The soluble fractions of CFE from cells grown in Ga-citrate and/or in media devoid of the metal (control) were subjected to BN-PAGE. A marked increase in SOD activity in cells grown in Ga-citrate was revealed. To better understand the nature of this enzymatic variation, the activity of SOD from soluble CFE of control and Ga-stressed cells at various growth intervals was investigated. Figure 6 shows a drastic increase in SOD activity in cells grown in the Ga-citrate media compared to cells grown in media devoid of the trivalent metal.

NADPH-generating enzymes

Since an increase in ROS and SOD activity were evident in Ga-stressed cells, it became clear that other enzymes than catalase were critical in countering this oxidative stress. Accordingly, cultures of *P. fluorescens* grown on citrate and Ga-citrate were harvested at various growth intervals and CFE were analyzed for NADPH-producing enzymes. The specific activities of G6PDH, 6-PGDH, ICDH–$NADP^+$ and ME were determined. The activities of G6PDH and ICDH–NADP were found to be elevated in the Ga-stressed cells, while 6-PGDH and ME activity did not appear to vary significantly. These data prompted the further investigation of the relationship of G6PDH and ICDH and the Ga-citrate induced stress. With the aid of BN-PAGE, the influence of Ga on ICDH activity was studied. Two activity bands were clearly evident in the Ga-stressed cells. The higher molecular mass band was absent in the control cells and higher activity observed in the lower band was due to higher protein expression. In addition, when cells grown in a medium containing Ga-citrate were transferred to a fresh medium devoid of the test metal the activity of this isoenzyme showed a drastic decline. Similarly, when cells grown in control media were transferred to a fresh medium containing H_2O_2 or menadione, the appearance of the ICDH isoenzyme was evident. Figure 7 indicated elevated levels of ICDH activity in the cells exposed to Ga-citrate. To gain further insight into this phenomenon the activity of this enzyme was monitored during various stages of growth with citrate or Ga-citrate serving as the sole carbon source. These studies revealed the specific activity of ICDH in the control (approximately 911 nmol NADPH produced min^{-1} mg $protein^{-1}$) remained relatively unchanged over a period of 30 h of growth. In contrast, cells grown in Ga-citrate exhibited an approximate 1.5-fold increase (approximately 1248 nmol NADPH produced min^{-1} mg $protein^{-1}$) and was not significantly altered during the growth period of 70 h. When *P. fluorescens* was subjected to a Ga supplemented media, a 60% increase in G6PDH activity was observed compared to control cells. This observation was further confirmed with the aid of BN-PAGE analysis of the soluble CFE from control and Ga-stressed cells isolated at late logarithmic phase of growth. Again, under Ga-stress, two more G6PDH isoenzymes were evident. These proteins were not discernable in the control cells (Figure 8, Table 1).

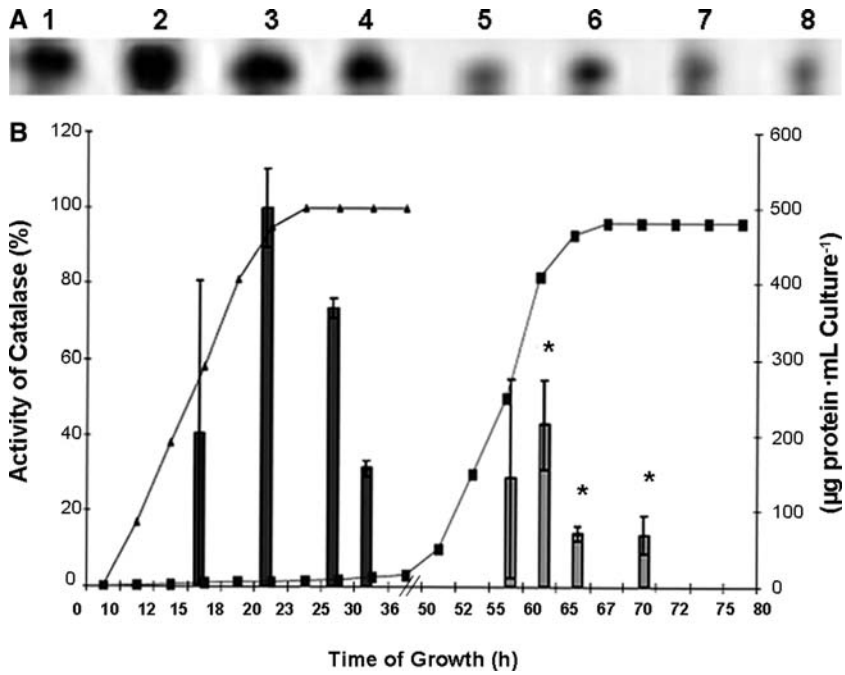


Figure 4. Panel A: BN-PAGE analysis of catalase activity in the soluble CFE. Lanes 1, 2, 3 and 4 correspond to control cells harvested after 15, 20, 25 and 30 h, respectively. Lanes 5, 6, 7 and 8 correspond to 1 mM Ga-stressed cells harvested after 55, 60, 65 and 70 h, respectively. 50 μg of protein were loaded in each lane. Panel B: Activity of catalase (H_2O_2 decomposition) in control (dark grey) and Ga-stressed (light grey) *P. fluorescens* soluble CFE at various growth intervals (▲ = control; ■ = Ga-stressed cells) $n = 3$). Values are means ± S.D. Catalase activity values differ significantly from controls (*) ($P \leq 0.05$). 100% = 8.93 μmol of H_2O_2 decomposed mg^{-1} protein min^{-1}.

Discussion

The data in this report clearly implicate Ga in the production of ROS. Although it has been postulated that this trivalent metal is a pro-oxidant and may contribute to an oxidative environment (Beriault 2004), this is the first demonstration of H_2O_2 production by CFE incubated with Ga-citrate. Hence, the toxicity of Ga appears to be due in part to its ability to generate this moiety *in vivo*. In fact, the increased lipid and protein oxidation observed in the Ga-stressed cells would favor this hypothesis. Furthermore, CFE subjected to Ga-citrate did generate H_2O_2 (Figue 2A). However, as this microbe is known to adapt to this toxic influence of Ga [26], it is conceivable that an anti-oxidative defense strategy was invoked. In this instance, it appears that the activation of SOD and two NADPH-generating enzymes helped mitigate the oxidative environment promoted by Ga. SOD is known to dismutate $O_2^{\bullet -}$ into H_2O_2 and O_2 and plays an essential role in ROS homeostasis (St Clair *et al*. 2005). The activity of the enzyme increased in cultures with Ga. Thus, it is quite likely that under Ga stress, an elevated level of $O_2^{\bullet -}$was produced. However, no increase in $O_2^{\bullet -}$was discernable by the INT assay in Ga cultures. It is within the realm of possibility that the

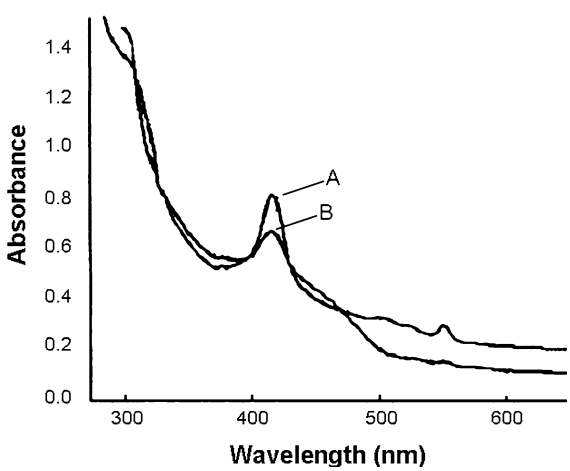


Figure 5. Detection of iron–sulfur (Fe–S) clusters in soluble CFE from (A) control cells and (B) Ga-stressed soluble fraction (note: equal protein concentration from cells obtained at the same growth phase were utilized). The peak at 395–420 nm have been shown to correspond to Fe–S clusters [4].

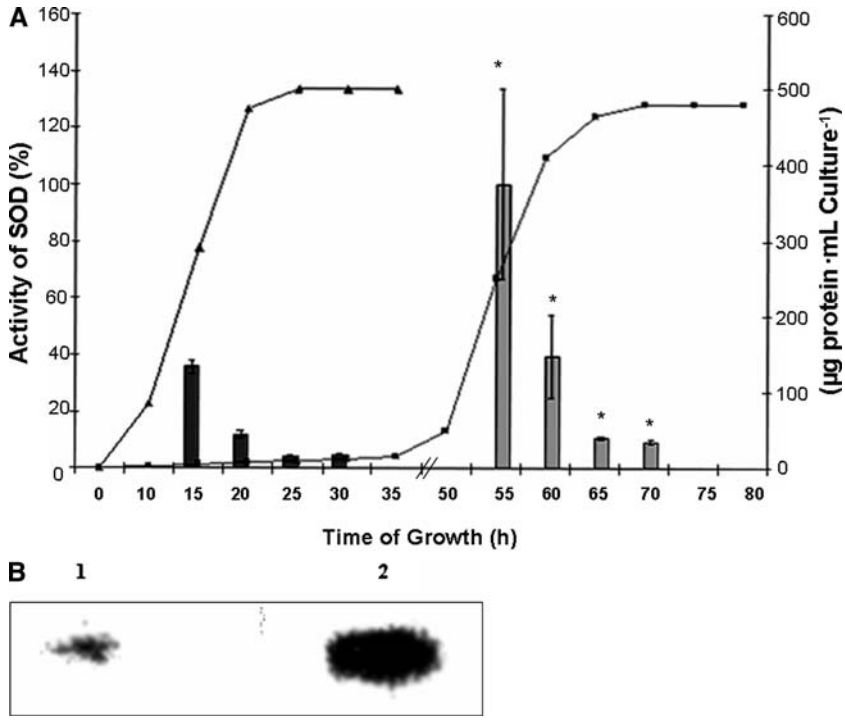
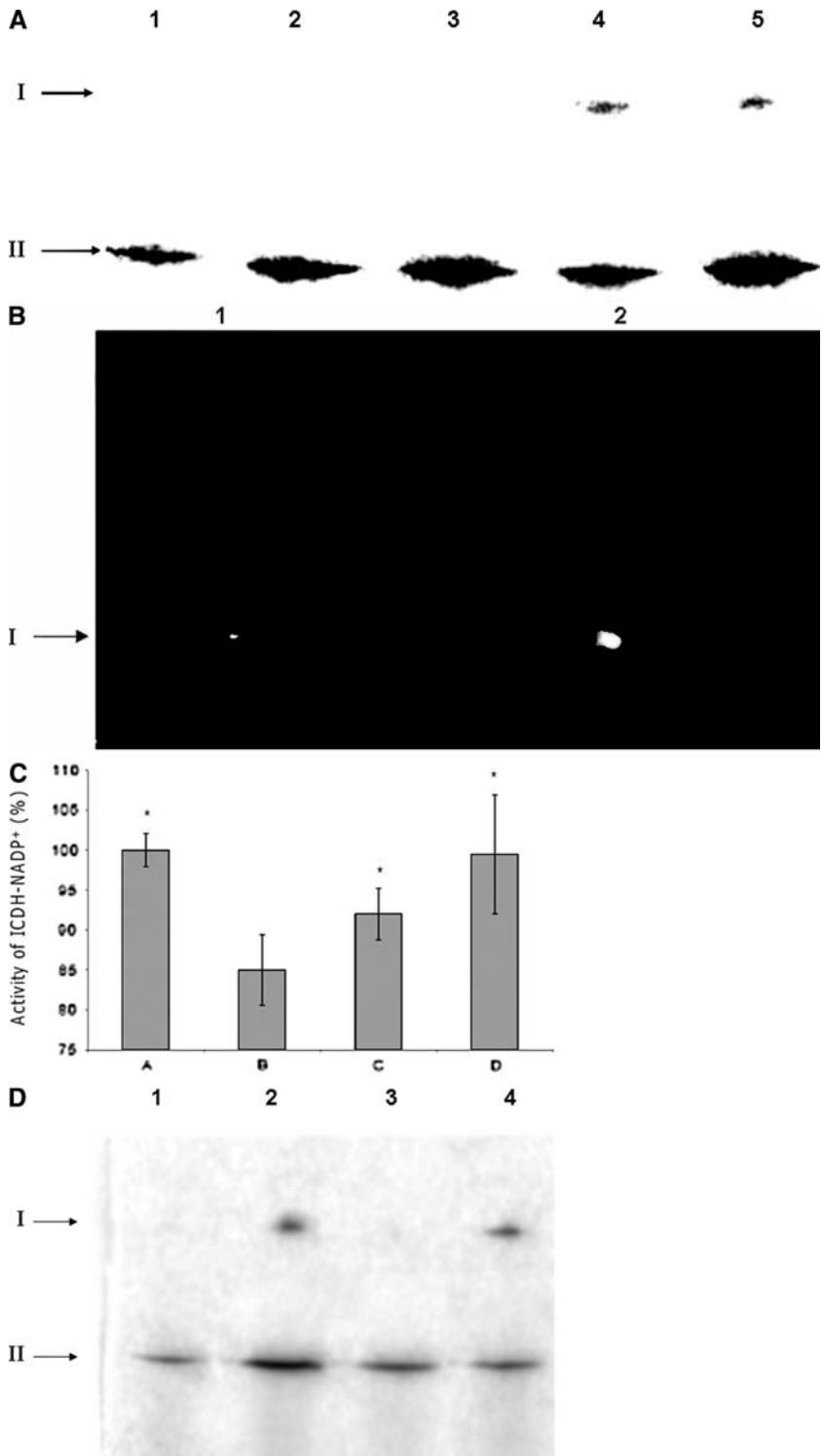


Figure 6. Panel A: Comparative study of SOD activity in control (dark grey) and in Ga-stressed (light grey) *P. fluorescens* membrane CFE at various growth intervals (▲ = control; ■ = Ga-stressed cells) ($n = 3$). Values are means ± S.D. SOD activity values differ significantly from controls (*) ($P \leq 0.05$). The activity of 55 h in the Ga culture (2.7 μmol of $O_2^{\bullet -}$decomposed mg^{-1} protein min^{-1} was taken as 100%). Panel B: BN-PAGE activity of SOD in control and Ga-stressed *P fluorescens* at logarithmic growth phase. Lane 1: cells grown in citrate medium (control). Lane 2: cells grown in Ga-citrate medium. About 30 μg of protein were loaded in each lane.

presence of this trivalent metal changes the speciation of $O_2^{\bullet -}$ and limits its detectability. The ESR data that revealed a significant change in the spectrum of $O_2^{\bullet -}$ in the presence of Ga would argue for such a postulation. It is important to note that although, Ga was able to quench the $O_2^{\bullet -}$ signal, it is not unlikely that it may interact with this ROS to generate more toxic species. It is evident from the data that the removal of Ga from the culture resulted in a decrease in SOD activity. When Ga-stressed cells were incubated in a control medium, a marked decrease of SOD activity was observed. We have recently demonstrated the pro-oxidative properties of another trivalent metal, Al (Singh *et al*. 2005).

The dependence of SOD activity on ROS has been demonstrated (Beyer *et al*. 1991). SOD has been shown to increase with increased respiration, a situation associated with elevated levels of $O_2^{\bullet -}$. Paradoxically, catalase an enzyme that degrades H_2O_2 a product of SOD was markedly diminished in the Ga-stressed cells. Ga is known to antagonize biochemical reactions necessitating Fe; hence, it is not unlikely that inference of Ga with Fe-metabolism would impede the genesis of enzymes or proteins requiring Fe. The soluble CFE from

Figure 7. (A) BN-PAGE analysis of ICDH–NADP activity in control and Ga-stressed *P. fluorescens* at various growth intervals. Lane 1, 2, 3: cells grown in citrate medium (control) for 15, 25 and 30 h, respectively. Lane 4 and 5: cells grown in Ga-citrate medium for 55 and 65 h, respectively. About 30 μg of protein were loaded in each lane. Note the appearance of an isozyme (Band I) in the Ga-stressed cells. (B) 2D BN-PAGE of ICDH–NADP+ in control and Ga-stressed *P. fluorescens*. Lane 1: control cells. Lane 2: Ga-stressed cells. (Note: the band II was loaded and protein level was detected by Coomassie staining.). (C) Influence of different effectors on ICDH–NADP activity in *P. fluorescens* ($n = 3$). A: Cells grown in 1 mM Ga-citrate for 65 h. About 10 mg of these whole cells were transferred for 6 h to media; B: (control media); C: (control media enriched with menadione); D: (control media enriched with H_2O_2). The specific activity in A is the mean ± S.D. of three independent experiments to which are compared the specific activity of B, C and D. ICDH–NADP activity values differ significantly from control (*) ($P \leq 0.05$). 100% = 1250 nmol NAPDH mg^{-1} of protein min^{-1} (D) In-gel activity staining of ICDH–NADP regulation with BN-PAGE. Lane 1: control cells grown in citrate medium. Lane 2: Ga-stressed cells. Lane 3: Ga-stressed cells into citrate medium. Lane 4: Control medium into Ga-stressed medium.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Activity of ICDH-NADP+ (%)
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II

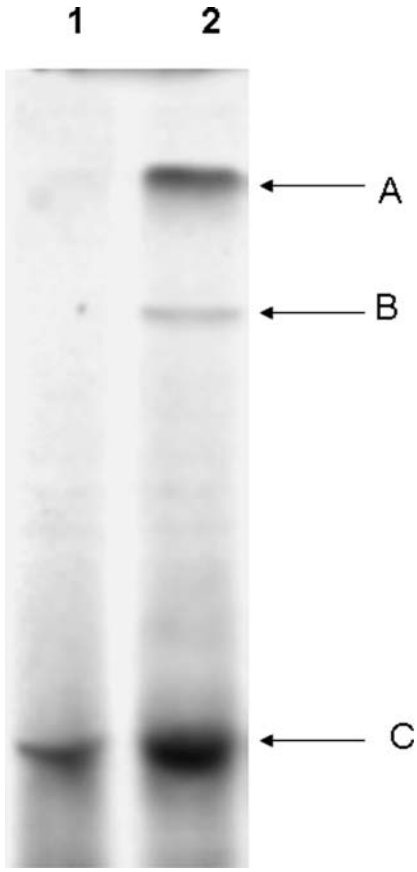

Figure 8. BN-PAGE analysis of G6PDH activity in control and Ga-stressed *P. fluorescens*. Lane 1: cells grown in citrate medium (control). Lane 2: cells grown in Ga-citrate medium (stress). About 60 μg of protein were loaded in each lane. Note the appearance of isoenzymes (Band A & B) in the stressed cells.

Ga-stressed cells yielded a markedly lower peak in the 395–420 nm region compared to the soluble CFE from control cells. This characteristic peak that is attributable to Fe–S cluster points for a dysfunctional Fe metabolism in the Ga cultures. This perturbation in Fe-metabolism provoked by Ga toxicity might have forced this organism to have decreased catalase activity; in fact, when Ga-stressed cells were either incubated in control cells or control cells supplemented with Fe, catalase activity was enhanced. Hence, decreased catalase activity observed in Ga-stressed cells might be due to the decreased bioavailability of Fe triggered by Ga stress. This diminution of catalase activity coupled with the increased expression of SOD would lead to an oxidative environment in the Ga-stressed cells and would result in severe inhibition of cellular growth. However, the physiological profile of *P. fluorescens* cultured in a Ga-medium showed that this organism adapted to this situation and that cell yield was relatively similar to that observed in control cultures (al-Aoukaty *et al.* 1992). Thus, it became important to evaluate other mechanisms that would help nullify this toxic influence of ROS and enable the organism to multiply. The homeostasis of NADPH, a crucial moiety that regulates the cellular redox-potential was the obvious choice. The four key enzymes that help fuel the synthesis of NADPH were evaluated.

Table 1. Specific activities of NADPH generating enzymes in control and Ga-stressed *P. fluorescens*

Enzyme	Specific activity (nmole NADPH produced min^{-1} mg $protein^{-1}$) in soluble fraction of CFE. Standard deviations are given in parentheses (n = 3).	
	Control cells	Ga-stressed cells
G6PDH	24 (± 2)	40 (± 3)
6-PGDH	2.0 (± 0.3)	2.6 (0.4)
ICDH	911 (± 30)	1248 (± 20)
Malic enzyme	172 (± 5)	168 (± 12)

A sharp increase in G6PDH and ICDH–NADP were observed. These enzymes were also characterized with novel isoenzymes that were absent or poorly expressed in the control cells. G6PDH has been widely regarded as an enzyme that contributes to NADPH production both for anti-oxidative defense and nucleic acid biosynthesis (Matsubara *et al.* 2001). In fact, numerous organisms subjected to ROS insult have been shown to overexpress G6PDH (Jung & Kim 2003; Randhir *et al.* 2005). The SoxR system that elaborates numerous proteins dedicated to protect a cell against $O_2^{\bullet -}$ does indeed include the gene for G6PDH. Hence, the overexpression and the production of isoenzymes in the Ga-stressed cells would contribute to decreased oxidative environment. G6PDH is critical both for cellular multiplication and anti-oxidative defense. When the H_2O_2 is too elevated, this enzyme has been shown to rectify this imbalance (Finzi *et al.* 2004). Hence, such a role for G6PDH in this instance is within the realm of possibilities. Indeed, the expression of the isoenzyme that was not detected in the control culture would strongly favor such an occurrence. In fact, when Ga-stressed cells were transferred to control cultures, the isoenzymes completely

disappeared and/or expressed in small quantities. ICDH–NADP, another key enzyme that generates NADPH, has only recently been identified as a pivotal participant in ROS defense. This enzyme, localized in the soluble cellular component, has been shown to have elevated activities in organisms subjected to oxidative stress (Middaugh *et al*. 2005). In fact, this protein has been demonstrated to be an important component of the peroxisomes, an organelle dedicated to the detoxification of H_2O_2 (Yoshihara *et al*. 2001; del Rio *et al*. 2002). The enzymes like catalase and glutathione peroxidase would be ineffective if they are not provided with an adequate supply of NADPH. And the presence of this enzyme in the peroxisome, an organelle whose primary mission is to detoxify H_2O_2, clearly indicates that an adequate level of NADPH is necessary to rectify the cellular oxidative imbalance. Only recently the effectiveness of this enzyme in combating oxidative stress is being understood. For instance, in *Plasmodium falciparum* the overexpression of ICDH–NADP enables this organism to survive the defense system of the host even in the absence of catalase (Wrenger & Muller 2003). It is also tempting to postulate that the α-ketoglutarate generated by the decarboxylation of isocitrate may also act as a potent antioxidant. Indeed, this α-ketoacid has been routinely utilized to relieve oxidative tension operative under various clinical situations (Nath *et al*. 1995). *In vitro*, ROS such as H_2O_2 is known to decarboxylate α-ketoglutarate with the concomitant production of succinate, H_2O and CO_2 (Yamamoto & Mohanan 2003). This process, unlike the enzymatic reactions involving catalase or glutathione peroxidase, does not necessitate the utilization of NADPH. Hence, the overexpression of ICDH–NADP would generate α-ketoglutarate and NADPH, two moieties that can participate in the diminution of the ROS stress promoted by Ga. In fact, it appears that α-ketoacids play a key role in H_2O_2 detoxification in organisms that lack catalase or peroxidase (Biagini *et al*. 2001).

In conclusion, this report demonstrates the ability of Ga to trigger an oxidative environment. This oxidative stress was countered by the overexpression of G6PDH and ICDH–$NADP^+$, two NADPH producing enzymes. The diminished activity of catalase due to the disturbance in Fe-metabolism provoked by Ga, raises the possibility of α-ketoglutarate as a potential ROS scavenger.

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