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ORIGINAL PAPER

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# Aluminum-tolerant *Pseudomonas fluorescens*: ROS toxicity and enhanced NADPH production

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**Abstract** Aluminum (Al) triggered a marked increase in reactive oxygen species (ROS) such as $O_2^-$ and $H_2O_2$ in *Pseudomonas fluorescens*. Although the Al-stressed cells were characterized with higher amounts of oxidized lipids and proteins than controls, NADPH production was markedly increased in these cells. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analyses coupled with activity and Coomassie staining revealed that $NADP^+$ -dependent isocitrate dehydrogenase (ICDH, E.C. 1.1.1.42) and glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) played a pivotal role in diminishing the oxidative environment promoted by Al. These enzymes were overexpressed in the Al-tolerant microbes and were modulated by the presence of either Al or hydrogen peroxide ($H_2O_2$) or menadione. The activity of superoxide dismutase (SOD, E.C. 1.15.1.1), an enzyme known to combat ROS stress was also increased in the cells cultured in millimolar amounts of Al. Hence, Al-tolerant *P. fluorescens* invokes an anti-oxidative defense strategy in order to survive.

**Keywords** Al toxicity · ROS · Isocitrate dehydrogenase · Glucose 6 phosphate dehydrogenase · Superoxide dismutase · NADPH

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## Introduction

Al, the most abundant metal in the Earth's crust, is not known to participate in any biological functions (Oteiza et al. 2004). However, its charge and its ionic radius enable this trivalent metal to mimic essential metals such as iron, magnesium and calcium. Al binds to iron-containing proteins and interferes with the metabolic pathways involved in energy production (Zatta et al. 2000). Al can readily replace Mg and bind ATP more strongly than Mg and perturbs numerous ATP-requiring metabolic processes (Exley et al. 1992; Koch et al. 2004). Calcium, an essential signaling element, is known to participate in a variety of biological processes. Al has been shown to interfere with calcium homeostasis and to interact directly with calcium binding enzymes (Kawano et al. 2003). Hence, the toxicity of Al has become a major cause of concern due to its increased occurrence in bio-available forms in the environment (Exley and Birchall 1992). Its role in various neurological disorders, in the abnormal development in fish and in the retardation of plant growth have been widely reported (Nayak 2002; Kawano et al. 2003).

The ability of metals like $Cu^{2+}$ and $Fe^{3+}$ to transfer electrons makes these metals one of the major culprits in the production of ROS in biological systems. If the concentration of these moieties is not properly controlled, ROS can lead to aberrant cellular activity and eventually death (Boonstra et al. 2004). Thus, it is not surprising that all organisms have devised intricate biochemical strategies to fend against the dangers posed by ROS. Anti-oxidants such as glutathione, enzymes such as SOD and proteins such as transferrin are some of the molecular strategies organisms deployed to maintain an intracellular reductive environment. These moieties are involved in the reduction, elimination and prevention of ROS production in vivo (Fridovich 1974).

Although Al has been proposed to act as a pro-oxidant due to its ability to increase the labile iron pool in

a cell and to participate directly or indirectly in the generation of ROS (Exley and Birchall 1992), an anti-oxidative response evoked as a consequence of Al stress has not yet been fully demonstrated. In this report, we show that *Pseudomonas fluorescens* subjected to millimolar amounts of Al do indeed experience oxidative stress. To ensure its survival, the organism overexpresses $NADP^+$ -dependent ICDH and G6PDH two enzymes known to promote a reductive environment. The role of SOD and catalase in the homeostasis of ROS in this Al-stressed microbe is also discussed.

## Materials and methods

### Microbial growth conditions and isolation of cellular fractions

The bacterial strain of *P. fluorescens* 13525 was obtained from the American Type Culture Collection (ATCC) and grown in a mineral medium containing $Na_2HPO_4$ (6.0 g); $KH_2PO_4$ (3.0 g); $NH_4Cl$ (0.8 g); $MgSO_4 \cdot 7H_2O$ (0.2 g), and citric acid (4 g) per liter of deionized water. Aluminum chloride was complexed to citric acid in a 1:1.26 i.e., 15 mM aluminum to 19 mM citric acid. Trace elements were present in concentrations as previously described (Anderson et al. 1992). The pH was adjusted to 6.8 with dilute NaOH. The media were dispensed in 200 ml amounts in 500 ml Erlenmeyer flasks, stoppered with foam plugs, and autoclaved for 20 min. at 121°C. The media were then inoculated with 1 ml of stationary-phase cells grown in a medium unamended with the test metal and aerated on a gyratory water bath shaker, model 76 (New Brunswick Scientific) at 26°C at 140 rpm. The Bradford protein assay was performed in order to determine the amount of cells in the culture by measuring solubilized cellular proteins, and bovine serum albumin was used as the standard (Bradford, 1976). The cells were treated with 1 M NaOH in order to afford the solubilized protein. The bacterial cells were harvested at various growth intervals and suspended in a cell storage buffer (pH 7.3) consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT. The cells were disrupted by sonication and centrifuged at 3,000 xg for 30 min. at 4°C to remove intact bacteria. Centrifugation at 180,000 xg for 2 h afforded a soluble CFE and a membrane CFE. These CFE fractions were kept at 4°C for up to 5 days and various enzymatic activities were monitored.

### Measurement of oxidized lipids, oxidized proteins, peroxide and superoxide

Oxidized lipids were quantitated by monitoring the amount of thiobarbituric acid reactive species (TBARS) as modified from (Kayashima et al. 2002). Briefly, 2 mg of protein equivalent of innermembranes were heated with 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25 M HCl in 1 ml for 15 min. Following centrifugation at 10,000*g* for 10 min, the supernatant was read at 532 nm. For the oxidized protein, the carbonyl content was determined with the aid of dinitrophenylhydrazine (DNPH) as described in (Vendemiale et al. 2001). Absorbance was recorded at 370 nm. An enzymatic assay involving peroxidase and p-anisidine was utilized to monitor the amount of $H_2O_2$ produced in cells with and without Al-stress (Yumoto et al. 2000). The CFE was incubated with citrate and Al-citrate for 30 min. respectively and absorbance was recorded at 458 nm (p-anisidine $\epsilon = 1.173\ M^{-1}\ cm^{-1}$). $H_2O_2$was utilized as the standard. The change in absorbance of iodonitrotetrazolium chloride (INT) at 485 nm was monitored in an effort to gauge the amount of $O_2^-$ generated in the CFE with citrate and Al-citrate as substrates respectively. Menadione was used as the standard (Fang et al. 2003).

### Monitoring of enzymatic activity and expression

ICDH, G6PDH, 6-phosphogluconate dehydrogenase (6PGDH) and malic enzyme (ME, EC 1.1.1.40) were analyzed by monitoring the amount of NADPH at 340 nm (Wynn et al. 1997). Isocitrate, glucose 6-phosphate, 6-phosphogluconate and malate were the respective substrates. The co-factor was $NADP^+$ and the reaction was performed in 25 mM Tris-HCl buffer (pH 7.3) with 5 mM $MgCl_2$, 0.1 mg protein $\cdot$ $ml^{-1}$ of the soluble CFE for 15 min. For ICDH and ME, the keto-acids, α-ketoglutarate and pyruvate was also quantitated with the aid of 2,4, DNPH at 450 nm (Romanov et al. 1999). For ICDH activity, 4 mM malonate was added in order to inhibit isocitrate lyase (ICL, EC 4.1.3.1). Controls consisted of the reaction mixtures without the CFE and substrates respectively. BN-PAGE were performed as described in (Schagger et al. 1991) with the following modifications. Sixty micrograms of proteins from the soluble CFE were applied to a 4–16% gradient gel, gently overlaid with blue cathode buffer, and subjected to electrophoresis. Immediately after electrophoresis, the gels were incubated with $H_2O_2$ (35 mM), p-anisidine (10 mM) in a reaction buffer pH 7.3, consisting of Tris HCl (25 mM) and $MgCl_2$ (5 mM). The degradation product of catalase reacts with p-anisidine to give a coloured band at the site of the enzymatic activity. For the visualization of SOD, the gel was incubated in the same buffer with INT (0.5 mg $ml^{-1}$) and menadione (15 mM). The appearance of an achromatic band was indicative of SOD activity (Caldwell et al. 2002). The membrane fraction was solubilized with 10% dodecylmaltoside prior to loading into the gel. CFE obtained from the same growth phases in the control and Al-stressed cultures were utilized in experiments where enzymatic activities were compared (Appanna et al. 1995).

### Analyses of ICDH and G6PDH activity and expression by BN-PAGE

In this instance, a 10–16% linear gradient gel was selected and formazan precipitation was utilized to visualize the two enzymes. The gels were incubated in the Tris-buffer as described above with INT (4 mg/ml), phenazine methosulfate (PMS) (4 mg/ml) and $NADP^+$ (0.5 mM). Isocitrate (2 mM) and glucose 6-phosphate (2 mM) were the substrates for ICDH and G6PDH respectively. Following the identification of the ICDH band, 2D and 3D BN-PAGE were also performed in the same conditions.

### ICDH and G6PDH activity and expression at various growth intervals and as a function of Al concentration

To evaluate the influence of Al on ICDH and G6PDH expression, the cells were grown at various growth intervals and following the isolation of the soluble CFE, the enzymes were monitored by BN-PAGE. Soluble CFE obtained from media with 0 to 15 mM Al was also analyzed for ICDH and G6PDH activity.

### Regulation of ICDH and G6PDH activity and expression

10 mg protein equivalent of cells isolated from control cultures were transferred to media with Al (15 mM), Al (15 mM) and rifampicin (50–200 μg/ml), Al (15 mM) and choramphenicol (50–200 μg/ml) respectively. Following 6–15 h of incubation, soluble CFE extracts were isolated and enzymatic activities were measured colorimetrically and by BN-PAGE (Wynn et al. 1997; Romanov et al. 1999).

## Results and discussion

Al-stressed cultures had at least threefold more oxidized lipids than control cultures (Fig. 1). The levels of oxidized proteins were higher in cells obtained in the Al-supplemented media than in the control media. A value of $0.05 \pm 0.002$ pmol carbonyl per mg protein was recorded in the Al-stressed cultures while in the control culture, only $0.025 \pm 0.004$ pmol carbonyl per mg protein was observed (data not shown). The presence of Al in the medium appeared to have triggered enhanced production of ROS. For instance, both in the CFEs from control and Al-stressed cells, $H_2O_2$ was sharply increased when Al-citrate was the substrate. The $O_2^-$ level in the Al-stressed cells was 12-fold higher in the presence of Al-citrate as the substrate, than when citrate was the substrate (Fig. 2). In the Al-stressed cells, the NADPH-generating enzymes were significantly more active. ICDH and G6PDH were characterized by the presence of isoenzymes that were more prominent in the Al-stressed cells (Table 1). The increase in ICDH activity was due to higher concentration of the enzyme in the Al-stressed cells. BN-PAGE and 3D BN-PAGE/SDS experiments indeed revealed that the soluble CFE of the Al-stressed cells was characterized by an increased amount of protein attributable to ICDH (Fig. 3). The increase in ICDH activity was time-dependent and was also sensitive to the amount of Al in the growth media. The maximal activity was observed at 30 h and 35 h of growth in 15 mM Al supplemented culture (Fig. 4) while significant augmentation of ICDH activity was evident in media with 1 mM or more of Al (Fig. 5). When control cells were incubated in a fresh Al media an increase in ICDH activity was noted. Furthermore, the isoenzyme was more prominent. However, when control cells were transferred to Al media with rifampicin or chloramphenicol, no significant change in ICDH profile was evident (Fig. 6). The ICDH activity in the control cells transferred in media with either $H_2O_2$ or menadione showed a similar pattern observed with the Al media.

G6PDH was also characterized by a sharp increase in activity in the Al-stressed cells. In this instance, the activity was localized by the appearance of three distinct bands on the gel, thus indicating the presence of three possible isoenzymes in the Al-stressed cells. Only two of these bands with markedly diminished intensities were observed in the cells isolated from the control media. The specific activity of this NADPH-generating enzyme was dependent on the presence of Al in the growth medium. A noticeable increase was evident when 5 mM or more Al was present in the culture (data not shown). The maximal activity in the 15 mM medium was observed at 35 h of incubation (Fig. 7). The expression of G6PDH appeared to be transcriptionally regulated. When control cells were subjected to Al-media with rifampicin or chloramphenicol, the bands corresponding to this enzyme were sharply diminished or absent.

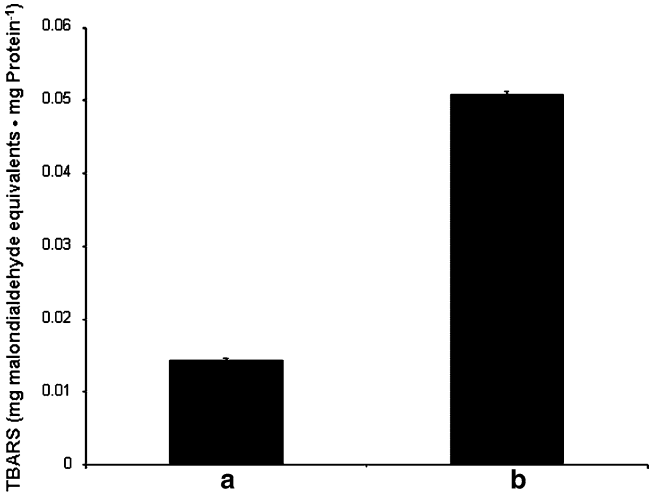


**Fig. 1** Oxidized lipids in Control and Al-stressed *P. fluorescens*. (200 ml cultures at late logarithmic phase were analyzed) **a** Control culture, **b** Al-stressed culture. ($n = 3 \pm$ SD)

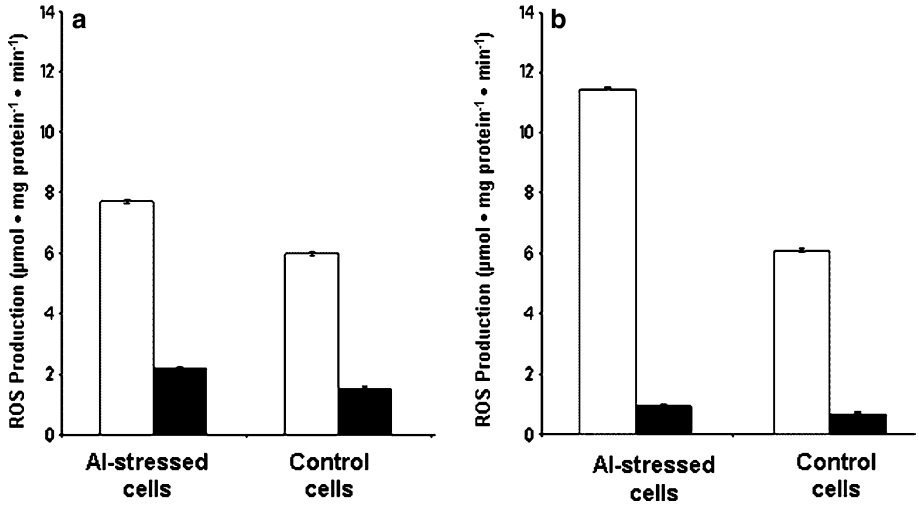


**Fig. 2** $H_2O_2$ and $O_2^-$ production by soluble CFE of *P. fluorescens*. Cells from late logarithmic phase were taken. Transparent bars represent Al-citrate as the substrate and dark bars represent citrate as the substrate. Panel **a**, $H_2O_2$ production. Panel **b**, $O_2^-$ production. ($n = 3$ mean ± SD)

**Table 1** Activity of NADPH generating enzymes in cells harvested from Al-stressed and control media at late logarithmic phase of growth. ($n = 3$, Mean ± SD)

| Enzymes | Specific activity (nmol $min^{-1}$ mg $protein^{-1}$) | |
|---|---|---|
| | Al-stressed cultures | Control cultures |
| ME | 82 ± 3 | 77 ± 5 |
| ICDH | 54 ± 2 | 35 ± 3 |
| G6PDH | 29 ± 6 | 17 ± 2 |
| 6-PGDH | 6.9 ± 0.3 | 4.6 ± 1.4 |

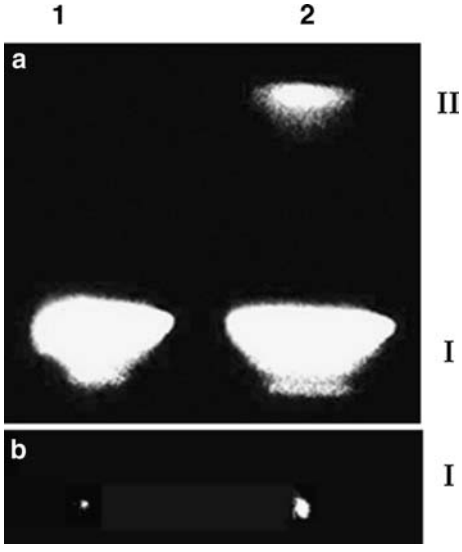


**Fig. 3** Effect of Al on ICDH activity and protein expression. **a** 1D BN PAGE activity stain for ICDH; **b** 3D BN PAGE/SDS Coomassie stain for ICDH. *1* Control soluble CFE. *2* Al-stressed soluble CFE (I and II depict isoenzymes of ICDH). Note: cultures at same growth phases were utilized

However, when the same control cells were incubated in an Al-medium without these inhibitors, three bands with higher intensities than the control cells were observed (Fig. 8). SOD an enzyme known to combat $O^-_2$ was markedly increased in the Al-tolerant cells. BN-PAGE analyses indicated a band indicative of SOD activity in the soluble CFE that was absent in the control cells. The band showing activity in the membrane CFE for the Al-stressed cells was more intense than in that from membrane CFE from the control cells. Catalase activity on the other hand, was not discernable in the cells isolated from Al-supplemented cultures (Fig. 9).

The data in this report point to an oxidative stress generated by Al and demonstrate how NADPH plays an instrumental role in diminishing the oxidizing environment promoted by this trivalent metal in *P. fluorescens*. Although increased lipid and protein oxidation were observed in the Al-exposed cells, the bacteria appeared to survive this deleterious situation by upregulating the activities of $NADP^+$ -dependent ICDH and G6PDH. This is the first demonstration of the production of such ROS as $H_2O_2$ and$O_2^-$ in CFE and an anti-oxidative defense evoked by Al. Recently, we have shown that this oxidative environment may be responsible for an ineffective aconitase in Al-stressed *P. fluorescens* (Middaugh et al. 2005).

It appears that the production of NADPH is markedly increased in the Al-stressed cells. The cytoplasmic ICDH, that is $NADP^+$ -dependent was augmented in the *P. fluorescens* subjected to Al. Indeed, an isoenzyme that was barely noticeable in the control cultures was also expressed. The expression of this enzyme increased with the level of the toxic metal in the culture medium. $NADP^+$ -dependent ICDH has been shown to play an important role in the reduction of an oxidative environment. In the malaria causing parasite, *Plasmodium falciparum*, both the mRNA transcript and the protein levels of this enzyme are upregulated (Wrenger et al. 2003). It is postulated that this biochemical adaptation enables this organism to survive the oxidative defense mechanism of the host. $NADP^+$ -dependent ICDH has also been shown to protect macrophages from ROS while in *S. cerevisiae*, the cytoplasmic variant of the enzyme has the highest level of defense against oxidative

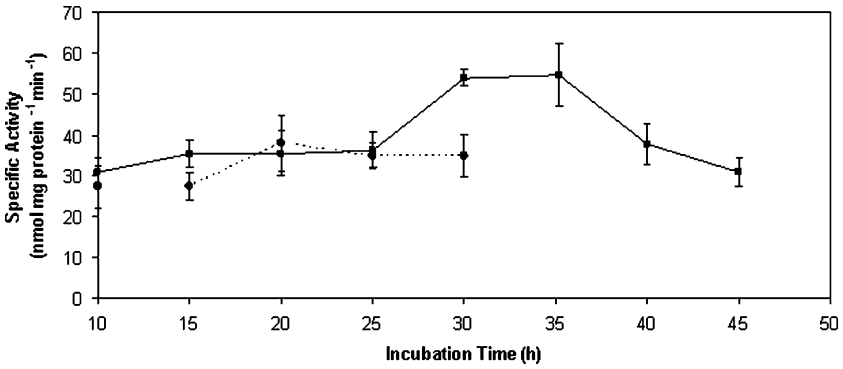


**Fig. 4** Specific Activity of ICDH during growth of *Pseudomonas fluorescens* on citrate (*circle*) and Al-citrate medium (*square*). ($n = 3$, Mean ± SD). (Cultures from Al-citrate media lag at least 10 h from those in citrate media). (Appanna et al. 1995)

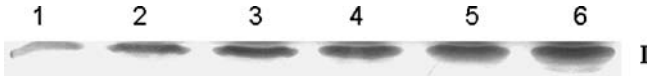


**Fig. 5** Influence of Al-stress on ICDH activity. Response of ICDH activity to increasing concentrations of Al. Lane 1, 0 mM. Lane 2, 0.1 mM. Lane 3, 1.0 mM . Lane 4, 5.0 mM. Lane 5, 10 mM. Lane 6, 15 mM. Only the low molecular weight isoenzyme is shown. (Isoenzyme I was monitored). CFE were obtained at the same growth phases (Appanna et al. 1995)

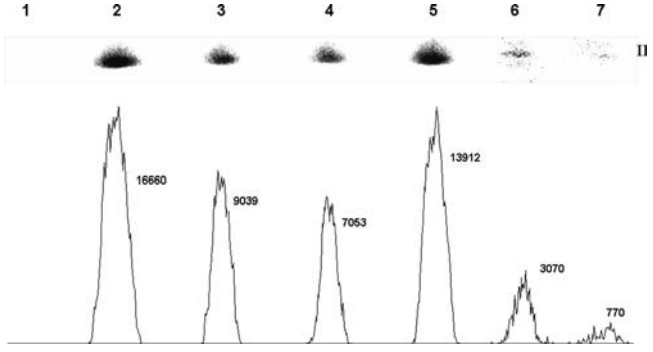


**Fig. 6** Influence of Al-stress on ICDH activity. *Lane 1* Control, *Lane 2* Al-stressed, *Lane 3* Control cells (late logarithmic phase) transferred to Al-stressed media, *Lane 4* Control cells transferred to media containing menadione, *Lane 5* Control cells transferred to media containing $H_2O_2$, *Lane 6* Control cells transferred to media containing Al and rifampicin, *Lane 7* Control cells transferred to media containing Al and chloramphenicol. (Isoenzyme II was monitored). (The CFE were obtained after 6–8 h of incubation in the stressed media). Band intensities for activity stain were measured using Scion image software (SCION Corporation, Frederick, MD)

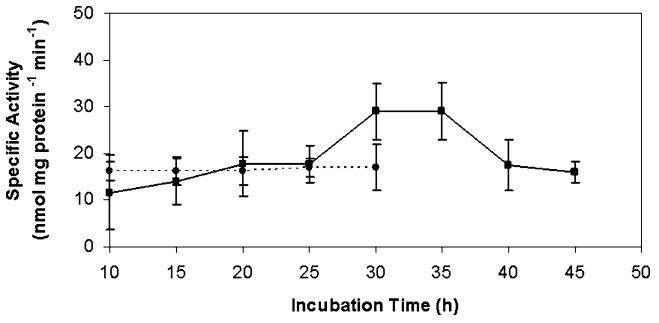


**Fig. 7** Specific Activity of G6PDH during growth of *P. fluorescens* on citrate (*circle*) and Al-citrate medium (*square*). ($n = 3$, Mean ± SD). (Cultures from Al-citrate media lag at least 10 h from those in citrate media). (Appanna et al. 1995)

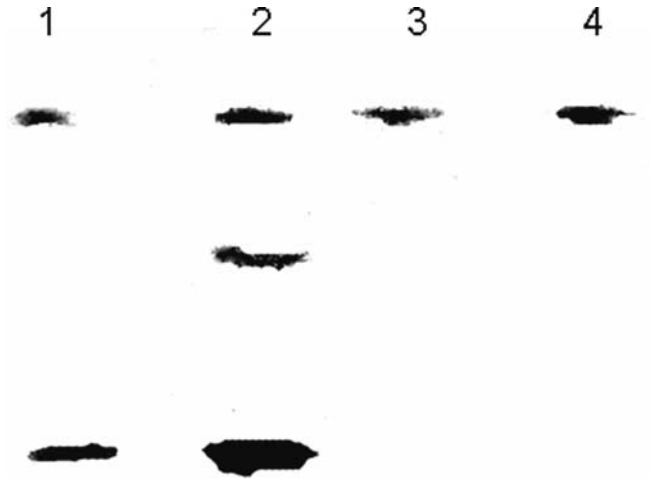


**Fig. 8** Influence of Al stress on G6PDH activity. G6PDH isoenzymes in *P. fluorescens* subjected to Al stress. *Lane 1* Cells grown in citrate media (*control*), *Lane 2* Control cells transferred to Al-citrate media, *Lane 3* Control cells transferred to Al-citrate media containing rifampicin. *Lane 4* Control cells transferred to Al-citrate media containing chloramphenicol. Cells in the same growth phase were compared (Appanna et al. 1995). In transfer experiments, cells were incubated for 6–8 h

stress (Contreras-Shannon et al. 2004; Maeng et al. 2004). In this study, when control cells were transferred to media with either menadione or the $H_2O_2$, the ICDH activity was similar to that observed in the Al media. However, when the Al-stressed cells were transferred to control media, the activity reverted back to normal levels. This would clearly call for for an important role of this enzyme in ROS defense in Al-stressed cells. The notion that α-ketoglutarate generated during this process may also act as an ROS scavenger may also be possible (Beriault 2004). G6PDH, another NADPH is also overexpressed in Al-stressed cells and is characterized by the expression of two isoenzymes. These proteins are either absent or minimal expressed in the control cells. G6PDH-deficient cells have been reported to show an increased propensity to oxidant induced senescence

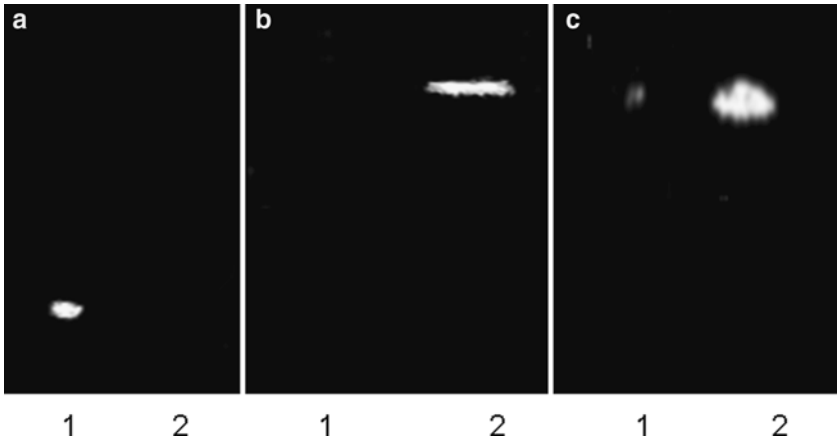


**Fig. 9** BN PAGE analysis of Catalase and SOD activities in Al-stressed and control CFE from *P. fluorescens*. **a** In-gel staining of Catalase *1* Control CFE, *2* Al-stressed CFE, **b** In-gel staining of soluble SOD. *1* Control CFE, *2* Al-stressed CFE, **c**: In-gel staining of membrane SOD, *1* Control membrane fraction, *2* Al-stressed membrane fraction. Cells in the same growth phase were utilized (Appanna et al. 1995)

(Kermici et al. 1990; Sailaja et al. 2003; Cheng et al. 2004), while in glucose-fed mice, the overexpression of this enzyme has been reported to promote increased lipogenesis (Amir-Ahmady et al. 2001). It is interesting to note that *P. fluorescens* undergoes enhanced lipid production when confronted with Al (Hamel et al. 2003). Hence, it is not unlikely that the increased synthesis of NADPH may have the dual purpose of combating oxidative stress and promoting lipogenesis. We have indeed demonstrated the overexpression of ICL in Al-stressed microbe. This enzyme that cleaves isocitrate to glyoxylate and succinate appears to be a pivotal contributor to oxalogenesis, a feature that enables this microbe to detoxify Al (Hamel et al. 2004). The activity of SOD, an enzyme that detoxifies $O_2^-$ was markedly enhanced in both soluble and membrane components of the Al-stressed cells. Since SOD exists predominantly in association with Cu, Zn or Mn, it is quite likely that Al does not severely impede the genesis of this enzyme (Fridovich 1974). On the other hand, catalase, an Fe-dependent enzyme was sharply lower. As Al is known to interfere with Fe uptake mechanisms (Exley and Birchall 1992), it is quite conceivable that a dearth of Fe in the Al-stressed cells would severely limit Fe-demanding processes and inhibit the production of Fe proteins such as catalase. Hence, in an effort to assure survivability in Al-stressed environment, *P. fluorescens* may invoke the use of α-keto acids like α-ketoglutarate, a product of ICDH and/or non-Fe dependent $H_2O_2$ degrading enzymes. The increased in expression of ICDH may help fulfill this dual purpose.

In conclusion, we demonstrate that Al triggers the production of such ROS as $H_2O_2$ and $O_2^-$ in *P. fluorescens*. The oxidative environment generated by Al is accompanied by the overexpression of $NADP^+$ - dependent ICDH and G6PDH. The enhanced production of NADPH plays a pivotal role in the adaptation of this microbe to Al stress. It provides a reductive environment to counter the oxidative stress imposed by Al and may contribute to increased lipogenesis, a feature critical in the sequestration of the trivalent metal. The possible involvement of α-ketoglutarate, the other product of ICDH in the detoxification of $H_2O_2$ in the Al-tolerant microbe is currently in progress.

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