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Overexpression of isocitrate lyase is an important strategy in the survival of *Pseudomonas fluorescens* exposed to aluminum

Robert Hamel,[a] Vasu D. Appanna,[a,*] Thammaiah Viswanatha,[b] and Simone Puiseux-Dao[c]

[a] *Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ont., Canada*
[b] *Department of Chemistry, GWC², University of Waterloo, Waterloo, Ont., Canada*
[c] *Toxicologie Environnementale, Museum National d'Histoire Naturelle, 12 rue Buffon, Paris 75005, France*

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Abstract

Isocitrate lyase, ICL (EC 4.1.3.1), an enzyme that cleaves isocitrate into succinate, and glyoxylate appears to play a pivotal role in the detoxification of aluminum (Al) in *Pseudomonas fluorescens*. Here, we present evidence that the 4-fold increase in ICL activity observed in Al-stressed cells is due to the overexpression of this enzyme. Blue-Native-PAGE, Western blotting, and spectrophotometric experiments revealed that ICL is optimally expressed at 35 h of growth in Al-stressed cells. However, following the immobilization of Al, at 60 h of growth, the level of the enzyme decreases markedly. This enzyme that exists as a homotetramer with a molecular mass of ~133 kDa appears to be transcriptionally regulated. The overexpression of ICL may be a specific response to Al-stress as *P. fluorescens* grown in the presence of such metals as Ga^{3+}, Pb^{2+}, and Ca^{2+} does not undergo any significant increase in ICL activity. Thus, these findings support the notion that the overexpression of ICL plays a pivotal role in the survival and in the increased oxalogenesis observed in Al-stressed *P. fluorescens*.

Keywords: Aluminum; Isocitrate lyase; Citrate metabolism; Oxalogenesis

Aluminum (Al) constitutes 7% of the earth's crust where it mainly occurs as biologically innocuous oxides and aluminosilicates. Acid rain, industrial wastes, and anthropogenic activities have led to a sharp increase in bioavailable Al. This bioavailability has become a major cause of concern as Al is known to be toxic to most living systems due to its ability to trigger the formation of reactive oxygen species (ROS), to interfere with iron metabolism, to interact with membrane lipids, and to mimic such metals as magnesium and calcium [1–5]. However, despite the toxicity associated with elevated levels of all metals, numerous organisms have evolved intricate biochemical mechanisms in an effort to adapt to metal-polluted environments. While Ca homeostasis may be attained by the deposition of this divalent metal as carbonate, an ATP pump invoked by organisms subjected to Cd has been reported [6,7]. Intracellular sequestration via polyphosphates and histidine does also provide an important route to metal immobilization [8,9]. Al-tolerance has been attributed to the sequestration of the metal within the anionic components of cell-walls and its compartmentalization in vacuoles [7]. Recently, the involvement of such organic acids as oxalic acid, malic acid, succinic acid, and citric acid in the deliberate exclusion of Al from normal cellular activities has also been reported. The overexpression of citrate synthase appears to be critical in organisms utilizing citrate as an Al-detoxifying metabolite. However, detailed biochemical mechanisms leading to the production of these organic acids as a consequence of Al-stress have not been shown [10,11]. In an attempt to elucidate the molecular detoxification pathway evoked in response to Al toxicity, we have uncovered a microbial model that grows on citrate, as the sole carbon source. This affords a unique system to probe the cellular interaction of Al. The involvement of oxalic acid and phosphatidylethanolamine (PE) in the immobilization of the trivalent metal was shown [12,13]. It appears that *Pseudomonas fluorescens* undergoes a complete shift in

* Corresponding author. Fax: +1-705-675-4844.
E-mail address: vappanna@laurentian.ca (V.D. Appanna).

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cellular metabolism in order to generate the precursors necessary for the biogeneses of oxalic acid and PE. The production of the dicarboxylic acid is generated via the oxidation of glyoxylate, a process that is markedly increased in the Al-stressed cultures. In this study, we provide evidence that ICL the key supplier of glyoxylate is overexpressed in cells subjected to Al. The transcriptional regulation of this process, its specificity to Al, and its involvement in oxalogenesis are also discussed.

Materials and methods

Organism and growth conditions. The bacterial strain *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. Trace elements were present in concentrations as previously described [6]. Aluminum chloride was complexed to citric acid in a 1:1.26 ratio, i.e., 15 mM aluminum to 19 mM citric acid. 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 waterbath 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 [14]. The cells were treated with 1 M NaOH in order to afford the solubilized protein.

Isolation of cellular fractions and ICL activity from P. fluorescens. Cellular fractions (outer membranes, inner membranes, cytoplasm, and periplasm) were isolated by the method described in [15], with the following modifications. The cells grown in the control and Al-stressed medium were harvested at different time intervals by centrifugation. The cells were separated from the insoluble pellet by repeated centrifugation at 100*g* and washing with 0.85% NaCl [16]. Cells corresponding to 1.5 g wet weight were suspended in 18 ml of ice-cold (20% w/v) sucrose and ice-cold reagents were added slowly to the suspension in an ice bath in the following order: 9 ml of 2 M sucrose, 10 ml of 0.1 M Tris–HCl, pH 7.8, 0.8 ml of 1% Na–EDTA, pH 7.0, and 1.8 ml of 0.5% lysozyme. The mixture was then warmed to 30 °C within a few minutes and kept at that temperature for 1 h. The suspension was centrifuged to remove the spheroplasts at 16,000*g* for 15 min at 30 °C. To recover the crude outer membranes, the supernatant was centrifuged at 45,000*g* for 60 min. The pellet, which contained the outer membranes, was resuspended in Tris buffer while the periplasmic components were in the supernatant. The spheroplasts were burst in 5 mM $MgCl_2$ and sonicated three times at intervals of 30 s. The inner membranes were recovered by centrifugation at 29,000*g* for 20 min and stored in Tris buffer. The supernatant yielded the cytoplasmic fraction. ICL activity was assayed in 25 mM Tris–HCl buffer, pH 7.3, containing 5 mM $MgCl_2$, 2 mM isocitrate, and approximately 0.1 mg/ml of protein equivalent from each cellular fraction, respectively. ICL activity was determined spectrophotometrically by monitoring the production of glyoxylate with 2,4,-dinitrophenylhydrazine (DNPH) at 450 nm as described in [17].

ICL expression as a function of Al concentration in the growth medium and in the presence of different metals. To elucidate the influence of Al on ICL expression, the cells were subjected to different concentrations of Al from 0 to 15 mM and ICL expression was monitored with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analyses. Furthermore, cells were grown in a citrate medium complexed to various metals such as Ga^{3+} (1 mM), Ca^{2+} (5 mM), Y^{3+} (0.1 mM), and Pb^{2+} (0.1 mM) and ICL expression was also investigated.

^1H NMR study of ICL activity from P. fluorescens. ^1H NMR analyses were performed using the Varian Gemini 2000 spectrometer operating at 200 MHz for ^1H. ICL was assayed in ^1H NMR buffer (10 mM phosphate, 5 mM $MgCl_2$, pH 7.4). ICL activity was studied as follows: the soluble fraction (200 μg protein equivalent) was incubated at 26 °C with isocitrate (2 mM) and the formation of succinate and glyoxylate was monitored. The ICL—catalyzed condensation of glyoxylate (5 mM) and succinate (2 mM) was also evaluated. The reactions were stopped by placing the tubes in a boiling water bath for 3 min. The formation of any precipitate was removed by centrifugation at 20,000*g* for 15 min. The supernatant was lyophilized and dissolved in 500 μl deuterium oxide (D_2O), 99.9 at.% D. The water resonance was suppressed with the aid of the homodecoupler set to the signal attributable to water. Experiments were executed with a 5 mm dual probe at a 90° pulse angle and 8 kbyte of data.

Electrophoretic gel analyses and staining techniques. SDS–PAGE was performed according to the method described by Laemmli [18]. The concentrations of the stacking gel and separating gel were 4% and 10%, respectively. The proteins from the soluble fraction were separated in a BN-PAGE as described by Schagger and Jagow [19] with the following modifications. Sixty milligrams of cytoplasmic protein was applied to a 4–16% gradient polyacrylamide gel gently overlaid with blue cathode buffer, and subjected to electrophoresis. Immediately, after electrophoresis, the gels were stained for protein content, and ICL activity.

For each set of gels, one gel was stained with Coomassie blue (0.25% brilliant blue R, 40% methanol, and 7% acetic acid) for 1 h and destained (10% acetic acid and 10% methanol) overnight. Other gels were used for determining enzyme activity by the following reactions. To measure ICL activity, the gel was incubated in 25 mM Tris–HCl containing 2 mM isocitrate, 5 mM $MgCl_2$, 0.5 mM NAD^+, 4 mg/ml iodonitrotetrazolium (INT), 4 mg/ml phenazine methosulfate (PMS), and 10 U/ml lactate dehydrogenase Rabbit type II (Sigma) [20]. Reactions were carried out at room temperature and stopped at various times by fixing the gel in 45% methanol and 10% acetic acid for 1 h. ICL activity was also measured with the use of a transilluminator. Photographs were taken with a digital imaging system utilizing a 5 s exposure. The staining solutions were freshly made. This solution was composed of equilibration buffer (50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, and 0.5 mM DTT) plus succinate (5 mM), glyoxylate (5 mM) $NADP^+$ (0.5 mM), and 2 U/ml IDH (Sigma) to a final volume of 10 ml. The gels were placed in the activity buffer and photographs were taken every 5 min. The fluorescence indicated the conversion of $NADP^+$ to NADPH resulting from the oxidation of isocitrate to α-ketoglutarate catalyzed by the addition of IDH. The isocitrate was produced via the condensation of succinate and glyoxylate from ICL localized in the gel.

Immunoblot experiments were performed on proteins resolved by SDS–PAGE. The proteins were transferred to HybondT-P (PVDF membrane) membranes, the detection of the desired antigen was achieved with the ECL Plus system (Amersham–Pharmacia Biotech). Polyclonal antibodies raised against ICL form *Mycobacterium avium* were generously supplied by Dr. K. Honer zu Bentrup, Cornell University, Ithaca, NY.

Results and discussion

ICL overexpression and characterization

ICL was predominantly localized in the cytoplasm and was inhibited by millimolar amounts of succinate. Its activity was also dependent on Mg^{2+}. With the aid of ^1H

NMR as a diagnostic probe, the nature of the enzyme was further confirmed. Signals corresponding to succinate (CH_2, 1.98 ppm) were evident in the reaction mixture obtained by incubating the cytoplasm with isocitrate. This signal was more prominent in the soluble fraction from Al-stressed cells (Fig. 1A). The signal for glyoxylate at 4.6 ppm was masked by the H_2O shift. However, the formation of isocitrate from succinate and glyoxylate was easily followed (Fig. 1B). In the control cytoplasm, the reactions were slower as the formation of isocitrate was barely discernable. Colorimetric analyses involving the measurement of glyoxylate formation with DNPH did confirm these findings. Precipitation with $(NH_2)SO_4$ and chromatographic analyses yielded ICL-rich samples. The specific activity in the Al-stressed cells was ~53 nmol/mg/min while the ICL activity from control cells had a value of ~11 nmol/mg/min. SDS–PAGE revealed a progressively enhanced band at 34 kDa after each purification step (Fig. 2). Western blot with the aid of ICL polyclonal antibodies did further attest to the nature of the bands as subunits of ICL. To further probe the enzyme, BN-PAGE was performed and the activity was detected with the aid of IDH and $NADP^+$. The formation of isocitrate from succinate and glyoxylate was detected by visualization of fluorescent NADPH and/or the formation of glyoxylate was probed with lactate dehydrogenase (LDH). A band corresponding to ~133 kDa was detected. In the Al-stressed cells at least a 4-fold increase in ICL band as quantified with the scion image software (Scion image version beta 4.0.2) was observed compared to the control cells. These bands reacted with the ICL antibodies and gave a singular band corresponding to 34 kDa by SDS–PAGE, thus revealing the homotetrameric nature of the enzyme (Fig. 3).

Aluminum concentration in the growth medium and ICL expression

In an effort to investigate the relationship between Al and ICL expression, cells grown in varying amounts of Al were harvested at stationary phase of growth. The ICL content in these cultures was monitored by SDS–PAGE and Western blot analyses. The level of ICL expressed in the cells increased drastically when the concentration of Al in the medium was in excess of 5 mM (Fig. 4). Although the levels of ICL did not increase over time during growth in 15 mM Al, a sharp decline was observed at 60 h of growth when most of the trivalent metal was sequestered as a PE and oxalic acid containing residue (Figs. 4 and 5).

Regulation and specificity of ICL expressed in Al-stressed P. fluorescens

In order to elucidate how the expression of ICL was regulated, cells obtained from Al cultures (A) at 35 h of

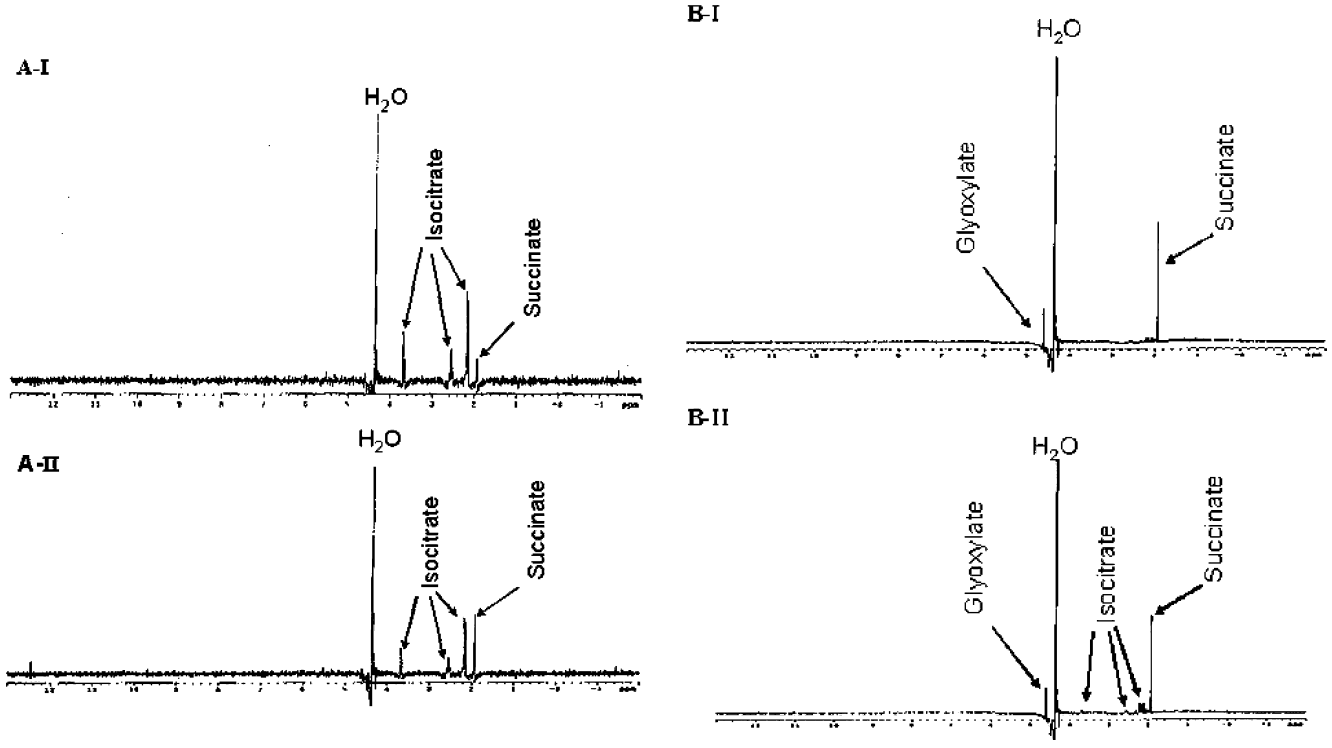

Fig. 1. ^1H NMR spectra of ICL activity from control (A-I, B-I) and Al-stressed (A-II), (B-II) *P. fluorescens*. A(I and II), isocitrate (2 mM) as substrate (forward direction). B(I and II), glyoxylate (5 mM), succinate (2 mM) as substrates (reverse direction). (Note the formation of isocitrate and succinate in Al-stressed cytoplasm.)

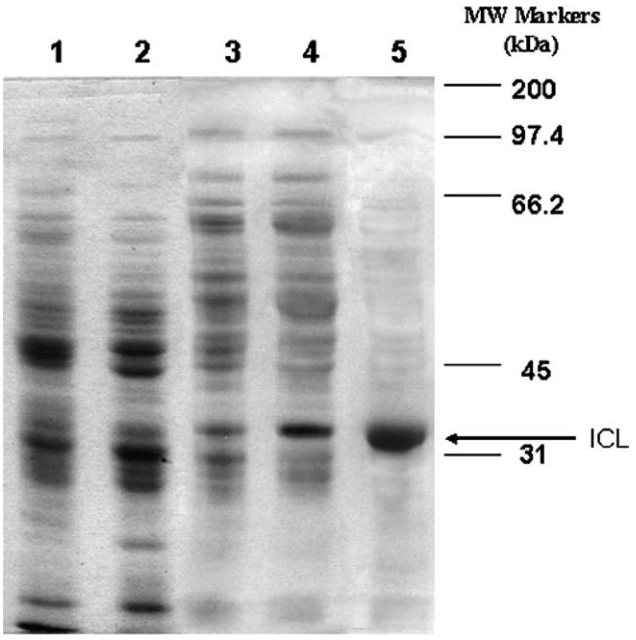

Fig. 2. SDS–PAGE of the purification profile of ICL. Lane 1, crude soluble fraction; lane 2, ultracentrifugation; lane 3, $(NH_4)_2SO_4$ precipitation; lane 4, DE 52 DEAE ion exchange chromatography; and lane 5, Sephadex G-200 chromatography. Molecular weight markers: Myosin (200 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), and ovalbumin (31 kDa).

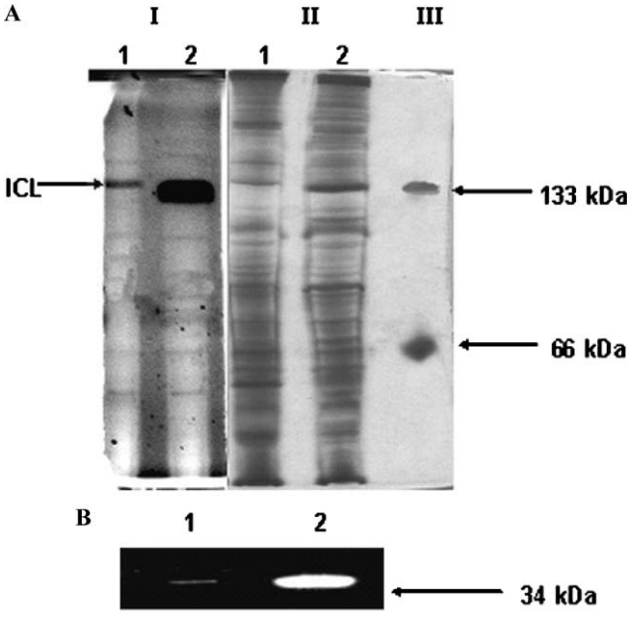

Fig. 3. (A) BN-PAGE:ICL activity staining and Coomassie staining. (I) Activity staining: lane 1, control; lane 2, Al-stressed. (II) Coomassie staining: lane 1, control; lane 2, Al-stressed. (III) Molecular weight markers. (B) Western blot analysis of control and Al-stressed soluble fraction following SDS–PAGE. Probed with polyclonal antibodies raised against ICL.

growth were transferred to a citrate medium devoid of Al. After 4 h of incubation, samples of the cells were transferred to an Al–citrate medium (B), an Al–citrate medium with rifampicin (C), an Al–citrate medium with chloramphenicol (D), and an Al–citrate medium without any inhibitors (E), respectively, and incubated for 6 h. The levels of ICL did experience a diminution in the control medium, while in the Al-medium, the expression of ICL underwent a sharp increase. In the Al–citrate medium with either of the two inhibitors, ICL levels were the same as in the control medium, thus indicating a transcriptional control of this isocitrate-degrading enzyme (Fig. 6). Furthermore, the increased expression of ICL appears to be specific to an Al-rich environment, as the presence of metals such as Ca^{2+}, Pb^{2+}, Y^{3+}, and Ga^{3+} did not trigger any discernable increase in ICL levels (Fig. 7).

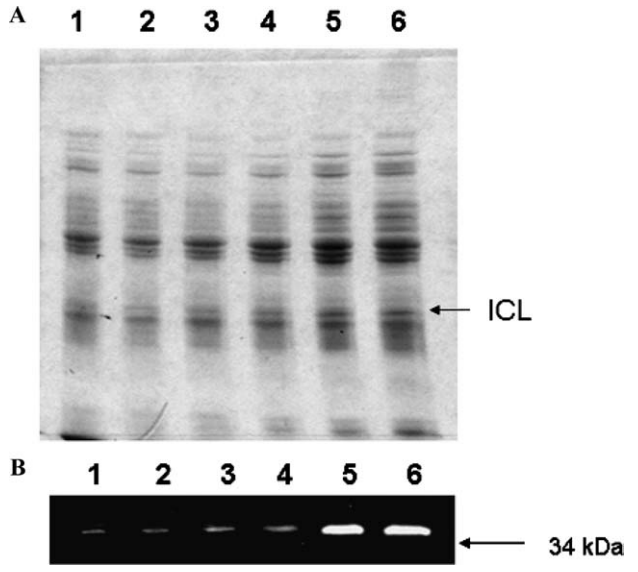

Fig. 4. Dependence of ICL expression on Al concentration in the growth medium. (A) SDS–PAGE, (B) Western blot analysis. Lane 1, no Al; lane 2, 0.1 mM; lane 3, 1.0 mM; lane 4, 5 mM; lane 5, 10 mM; and lane 6, 15 mM.

The results presented point to an Al-evoked overexpresssion of ICL in *P. fluorescens*. The level of ICL expressed is sensitive to the concentration of Al in the culture medium and declines following the immobilization of the trivalent metal in association with oxalic acid and PE. This is the first demonstration of enhanced production of ICL provoked by Al stress. ICL is known to catalyze the reversible cleavage of isocitrate into succinate and glyoxylate, the first committed step of the glyoxylate shunt [21]. Succinate enters the tricarboxylic acid cycle (TCA), while glyoxylate, via the enzyme malate synthase (MS), and acetyl CoA, is converted to malate. This detour from the TCA cycle effected by ICL allows an organism to bypass the two oxidative steps responsible for the production of the reducing factor, NADH. This carbon-conserving metabolic pathway also ensures that an adequate supply of TCA-cycle intermediates is channeled towards biosynthetic reactions. Hence, ICL contributes to anaplerotic reactions and

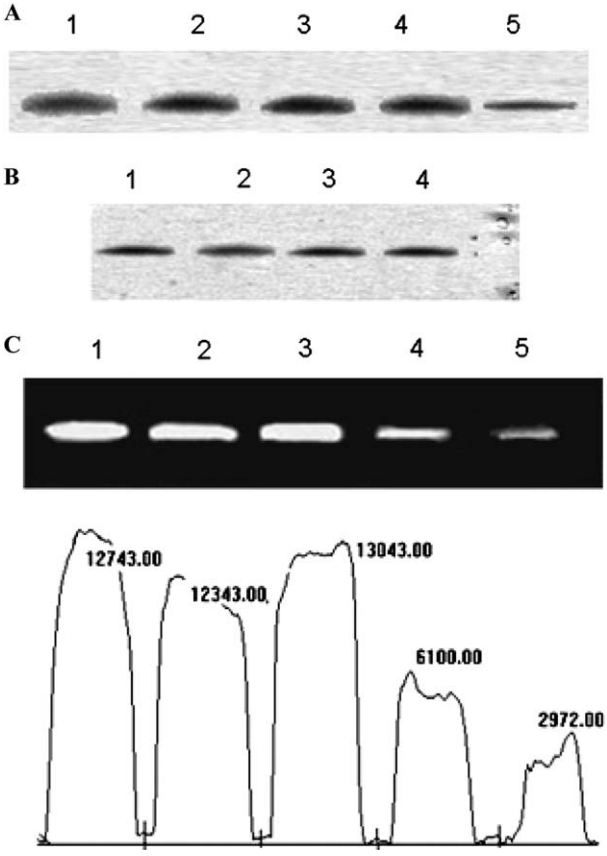

Fig. 5. ICL expression at various growth intervals. (A) BN-PAGE and activity staining (Al-stressed cultures). Lane 1, 20 h; lane 2, 30 h; lane 3, 35 h; lane 4, 45 h; and lane 5, 60 h. (B) BN-PAGE and activity staining (control cells). Lane 1, 15 h; lane 2, 20 h; lane 3, 25 h; and lane 4, 30 h. (C) Proteins resolved by SDS–PAGE were analyzed by Western blot analysis (Al-stressed cultures). Lane 1, 20 h; lane 2, 30 h; lane 3, 35 h; lane 4, 45 h; and lane 5, 60 h. Intensities were quantitated using scion image software. Relative area of the bands are given.

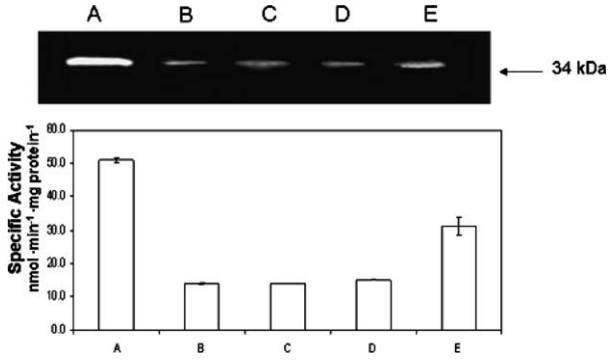

Fig. 6. Regulation of ICL. (A) Cells from Al–citrate medium; (B) cells from (A) incubated for 4 h in media devoid of Al (control media); (C) cells from (B) incubated (6 h) in fresh Al–citrate media containing rifampicin (200 μg/ml); (D) cells from (B) incubated (6 h) in fresh Al–citrate media containing chloramphenicol (200 μg/ml); and (E) cells from (B) incubated (6 h) in fresh Al–citrate media devoid of protein synthesis inhibitors.

allows numerous organisms to thrive in environments rich in two-carbon compounds and/or fatty acids [22]. Indeed, the prolific growth and pathogenicity of the

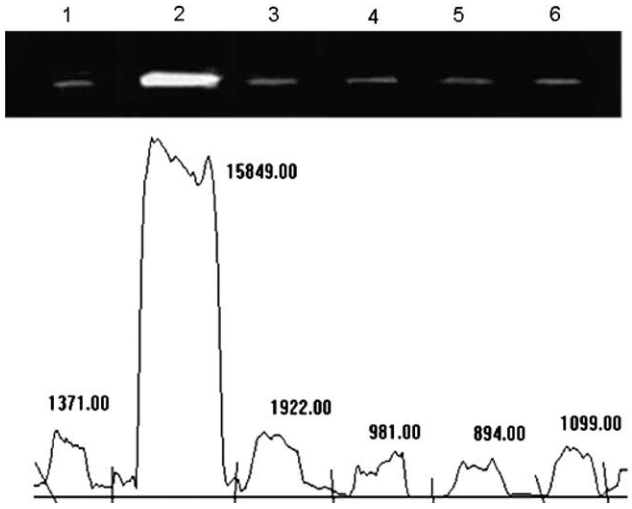

Fig. 7. ICL expression in *P. fluorescens* from cultures containing other metals; lane 1, control; lane 2, Al^{3+}; lane 3, Ga^{3+}; lane 4, Ca^{2+}; lane 5, Y^{3+}; and lane 6, Pb^{2+}. Western blot analysis. Intensities were quantitated using scion image software.

microbe *Mycobacterium* have been attributed to the ability of this organism to invoke the participation of ICL in its metabolism [23]. This isocitrate cleaving enzyme does also provide an evolutionary advantage to organisms confronted to an acetate medium and to seeds storing lipids as a major source of carbon. In the latter instance, ICL acts as a morphogen as it mediates cellular development [24]. In the present study, it appears that ICL contributes significantly to the production of glyoxylate, a metabolite that is required for oxalate production via a glyoxylate oxidizing enzyme. In fact, the latter process is increased 8-fold in Al-stressed cells [25]. It is important to note that MS, the other competitor of glyoxylate, has been shown not to be affected by Al stress. Thus, it is conceivable that this decoupling of the ICL from MS enables the funneling of glyoxylate towards the genesis of oxalic acid, a moiety that is critical for the detoxification of Al.

And as the overexpression of ICL is responsive to the concentration of Al in the growth medium, it is likely that the need for oxalate may be playing a pivotal role in regulating this process. As the demand for glyoxylate subsides following the immobilization of Al, the ICL is down-regulated. This process is sensitive to rifampicin, a compound known to interfere with transcriptional machinery. The regulation of ICL via the perturbation of the mRNA has been reported [20]. As the organism was cultured in a medium with citrate as the sole source of carbon, it is important that this tricarboxylic acid can be rapidly metabolized either via citrate lyase or aconitase. Since no citrate lyase activity [26] has been observed, it is very likely that aconitase may be providing the only route for the utilization of citrate. However, it has been well established that Al stress may result in iron deprivation [27]. Such a situation would likely lead to

decreased aconitase activity (V. Appanna, unpublished results) and an overexpression of ICL in an Al-rich environment may also facilitate citrate degradation by shifting equilibrium towards isocitrate formation. Hence, the overproduction of ICL may contribute to the survival of the organism in an Al–citrate environment.

In conclusion, we have presented evidence that clearly indicates that *P. fluorescens* invokes the overexpression of ICL in an effort to combat Al toxicity. The biogenesis of this homotetramer appears to be Al-sensitive and transcriptionally regulated. Although the overexpression of ICL in Al-stressed cells helps fuel oxalogenesis, it is quite likely that the enzyme diverts metabolites from the TCA cycle and also enables the degradation of citrate under this stressed situation. The delineation of these biochemical events and the global metabolic flux mediated by Al stress is currently underway.

Acknowledgments

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