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Aluminum detoxification in *Pseudomonas fluorescens* is mediated by oxalate and phosphatidylethanolamine

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Abstract

^{13}C NMR studies with aluminum (Al)-stressed *Pseudomonas fluorescens* revealed that the trivalent metal was secreted in association with oxalate and phosphatidylethanolamine (PE). These moieties were observed in the insoluble pellet obtained upon incubation of these resting cells in the presence of either Al-citrate or citrate. This extrusion process was concomitant with the utilization of either of these tricarboxylic acids as a substrate. While only minimal amounts of Al were secreted in the presence of such carbon source as glucose, succinate or oxaloacetate, oxalate did permit the efflux of Al. Neither α-ketoglutarate nor ethylenediaminetetraacetic acid (EDTA) was effective in dislocating Al from the cells. The elimination of Al from the cells did not appear to be affected by *p*-dinitrophenol (DNP) or dicyclohexylcarbodiimide (DCCD) or azide, but was sensitive to temperature, pH and cerulenin, an inhibitor of lipid synthesis. Thus, it appears that *P. fluorescens* detoxifies Al via its extrusion in association with oxalate and PE in a process that apparently does not necessitate the direct utilization of energy.

Keywords: Oxalate; Phosphatidylethanolamine (PE); Aluminum; Isocitrate lyase

1. Introduction

Although aluminum (Al) is a widely occurring metal, its insolubility assures its low bioavailability. However, acid rain, industrial wastes and anthropogenic activities have led to a sharp rise in the concentration of the biologically active forms of this metal. This situation has become a major cause of concern as Al is known to interfere with the normal functioning of most cellular systems. DNA, iron proteins and biochemical reactions mediated by calcium appear to be the primary target of the Al toxicity [1–3]. Despite the toxicity associated with elevated levels of metals, numerous organisms are known to elaborate intricate strategies in an effort to survive in metal polluted environments. Active efflux, intracellular sequestration, volatilization, precipitation and biotransformation of the metals are some of the mechanisms evoked to combat metal stress. While an ATP-dependent pump is involved in the removal of intracellular Cd^{2+}, lowering of cytoplasmic Ca^{2+} content is achieved via the deposition of the divalent metal as calcite [4,5]. Pumps utilizing either ATP or the proton-motive force to expel metal toxicants from the cells into the external environment are also known. The elimination of Zn^{2+} and Co^{2+} via a cation/proton antiporter have been shown. Unique biomolecules such as cysteine-rich metallothioneins and polyphosphates have been implicated in the sequestration of metals [6,7]. Recently, dicarboxylic and tricarboxylic derivatives such as oxalate, malate, succinate or citrate, which are known to tightly bind Al, have been reported to play an instrumental role in the deliberate exclusion of the trivalent metal from the normal functioning of various cellular systems [8–10].

In an effort to understand the biochemical responses elicited by Al, our laboratory has uncovered an interesting model system to probe the cellular interactions of this trivalent metal [11,12]. The Al complexed to citrate is provided as the sole carbon source to the soil microbe *Pseudomonas fluorescens*. We have demonstrated the ability of this microbe to tolerate millimolar amounts of Al. It appears that the trivalent element is detoxified via its insolubilization as a lipid-containing residue. Precipitation occurs following the initial entry of the Al in the cell. We have recently demonstrated an eightfold increase in the ability of Al-stressed cells to effect the production of oxalate via the oxidation of glyoxylate compared to control cells [8].

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Furthermore, the need for glyoxylate has been shown to trigger a major reconfiguration of various enzymes in an effort to marshal cellular metabolism towards the generation of this moiety [13]. In this report we describe the ability of Al-stressed *P. fluorescens* to secrete the trivalent metal in association with phosphatidylethanolamine (PE) and oxalate. This extrusion process seems to require neither a protonmotive force nor ATP hydrolysis. However, it is sensitive to cerulenin, an inhibitor of lipid synthesis [14]. The expulsion of Al by *P. fluorescens* with the participation of oxalate and PE appears to constitute a novel metal detoxification mechanism.

2. Material and methods

2.1. Materials

p-Dinitrophenol (DNP), cerulenin, dicyclohexylcarbodiimide (DCCD) and oxalate monitoring kit were from Sigma. The Bradford assay kit was purchased from Bio-Rad while the citrate assay kit was obtained from Boehringer. [2,4-$^{13}C_2$] citric acid was a product of ISOTEC, Miamisburg, OH.

2.2. Microbial strain and growth conditions

P. fluorescens ATCC 13525 was from the American Type Culture Collection and was maintained and grown on a mineral medium consisting of Na_2HPO_4 (6.0 g), KH_2 PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4.7H_2O$ (0.2 g) and citric acid (4.0 g) per litre of deionized distilled water. Trace elements in concentrations previously described were also included [5]. In the Al-stressed medium, citric acid was complexed to Al chloride in a molar ratio of 1.26:1, i.e. 19 mM citrate to 15 mM Al chloride. The pH was adjusted to 6.8 with dilute NaOH and the media were dispensed in 200 ml amounts 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. Bacterial multiplication was monitored by measuring solubilized protein at various incubation intervals with the aid of the Bradford assay [15].

2.3. Al-loaded P. fluorescens

Cells obtained at 40 h of incubation in Al media that have been shown to effect maximal Al uptake were utilized to load the *Pseudomonas* with the trivalent metal [16]. Briefly, cells corresponding to 1.5 mg of protein were incubated with Al-citrate (3:1 or 1:1 mM) for 30 min and washed three times with buffered saline (0.85%). The cells were digested in conc. HNO_3 and Al concentration was monitored by the aluminon assay [17]. This procedure afforded 9.1 ± 0.8 μg of Al mg^{-1} of cells (measured as protein equivalent, following the solubilization of the cells with NaOH). Similar treatments were performed on control cells and heat-inactivated Al-stressed cells. No significant intracellular accumulation of Al was discerned.

2.4. Measurement of Al secretion, citrate utilization, PE and oxalate formation

In experiments designed to measure Al efflux, the metal loaded cells (13.6 ± 1.2 μg Al 1.5 mg^{-1} of protein equivalent of cells) were incubated in 3 ml of 25 mM Tris-buffer (pH 7.0) at 26 °C in a water bath shaker. The reaction was initiated by introducing in the mixture varying amounts of citric acid or Al-citrate. The reaction mixture without the citrates served as controls. At timed intervals, the cells were separated by centrifugation or by filtration with a 0.22-μm millipore. The Al content was analyzed colorimetrically in the supernatant and in the cells following digestion in HNO_3. The supernatant that yielded a pellet upon spinning at $180{,}000 \times g$ for 2 h was also analyzed. Citrate was monitored by the enzyme coupled assay based on citrate lyase [18]. The phospholipid content in the supernatant was quantitated with the dye Victoria Blue R [19]. The supernatant obtained following the removal of the bacteria, was centrifuged at $180{,}000 \times g$ for 2 h and the pellet was resuspended and examined for its Al, PE and oxalate contents [17,19,20]. Citrate ranging from 0 to 10 mM was utilized and K_m was determined from the double reciprocal plot. The cells grown on citrate only did not show any Al translocating characteristic. Al-loaded cells were heated at 60 °C for 5–10 min and were subjected to the efflux assay with citrate or Al-citrate as substrate.

2.5. Effects of various inhibitors on Al extrusion

Cells corresponding to 1.5 mg of solubilized protein and 13.6 ± 1.2 μg Al^{3+} were incubated in the presence of an inhibitor for 30 min. The reaction was initiated by introducing citric acid in the mixture. No significant variation in efflux properties was observed in the presence or following the removal of the inhibitor prior to the addition of citric acid. Sodium azide (NaN_3), DNP, cerulenin and DCCD were in 10% acetone and their final concentrations were 5 mM, 4 mM, 450 μM and 1 mM, respectively. Acetone (10%) did not have any observable interference on Al efflux. Aluminum and citrate were monitored following the removal of the cells. All experiments were repeated twice and in triplicate.

2.6. Determination of Al efflux in the presence of various carbon sources

To investigate the specificity of this Al efflux system in *P. fluorescens*, various carbon sources were utilized. Cells (13.6 ± 1.2 μg Al 1.5 mg^{-1} protein) were incubated for 30 min in the presence of 10 mM of isocitrate, oxaloacetate, oxalate, malate, Al-citrate, α-ketoglutarate, glucose and tricarballylic acid, respectively. Ethylenediaminetetraacetic

acid (EDTA) (1 mM) was also utilized as a potential substrate. Following the removal of the cells, the Al in the supernatant was analyzed. The Al-loaded cells were heated to 60 °C and their Al-secreting characteristic was studied with citrate and Al-citrate as the carbon source. The ability of the cells to exude Al at 4 °C was also monitored. The effect of pH on this extrusion process was evaluated by initiating the efflux reaction in incubation mixtures with a pH range of 5.4 to 8.2.

2.7. Nuclear magnetic resonance (NMR) studies

NMR analyses were performed using a Varian Gemini 2000 spectrometer operating at 50.38 MHz for ^{13}C. The ^{2}H resonance of D_2O (5%) was used to lock the field and for shimming. Experiments were performed with a 5-mm broad-band probe (35° pulse, 1-s relaxation delay, 8 kilobytes of data and 2000 scans). Chemical shifts were referenced to shifts of standard compounds obtained under the same conditions. Intact cells (250 μg equivalent of solubilized protein) obtained after 40 h of incubation in an Al-citrate (15 mM) medium were placed in a phosphate buffer (10% D_2O). The reaction was initiated by addition of labeled Al-citrate [2,4-$^{13}C_2$] (1:1). Following 3-h incubation at 26 °C, the cells were centrifuged at 6000 × *g* in order to afford a cellular pellet and a supernatant. The supernatant was subsequently centrifuged at 180,000 × *g* and a soluble component and pellet fraction were obtained. The latter was extracted with $CHCl_3/CH_3OH/H_2O$ (2:1:0.8). All these various fractions were subjected to proton decoupled ^{13}C NMR analyses. The identities of the different peaks were confirmed by running the appropriate known standard compounds and by enzymatic assays utilizing lactate dehydrogenase for glyoxylate, oxalate oxidase for oxalate and succinate dehydrogenase for succinate respectively [13,21,22]. Similar experiments were also performed on cells grown in labeled Al-citrate and various fractions were subsequently analyzed.

3. Results and discussion

3.1. ^{13}C NMR studies of Al-citrate metabolism and Al efflux

The pellet obtained at 180,000 × g following the incubation of Al-stressed cells with Al-citrate [2,4-$^{13}C_2$] revealed peaks at 168 ,139, 123 and 99 ppm. The residue was treated with $CHCl_3/CH_3OH/H_2O$. The aqueous layer was found to contain only the 168 peak, a fingerprint of Al-oxalate [8]. While the organic layer showed peaks at 139, 123 and 99 ppm. The peaks at 139 and 123 ppm were attributable to the olefinic carbon of PE [23]. This moiety has been confirmed to be PE by thin layer chromatography and ^{13}C NMR of lipid obtained from a 2-l batch culture. A peak at 83 ppm in the soluble cellular fraction that was attributable to [2-$^{13}C_1$] of glyoxylate would imply the cleavage of the tricarboxylic acid contributed to the formation of this moiety. The resonance at 27 ppm was assigned to the [CH_2-$^{13}C_1$] of succinate. The peak at 158 has been attributed to free oxalate [8]. The 137 ppm in the soluble cellular fraction has not been assigned yet (Fig. 1). The presence of glyoxylate, oxalate and succinate was further confirmed by enzymatic assays utilizing lactate dehydrogenase, oxalate oxidase and succinate dehydrogenase, respectively.

3.2. Al efflux and citrate utilization

When Al-loaded cells were incubated in the presence of citric acid, Al was rapidly secreted. This process appeared to be relatively fast as 90% of the trivalent metal was localized in the supernatant fluid within 5 min of incubation. The apparent $K_{m(citrate)}$ to effect this reaction was 2.2×10^{-1} mM, while the rate of Al extrusion was 15 nmol mg^{-1} protein min^{-1} (Fig. 2). The secretion of Al was concomitant with citrate utilization and the formation of extracellular PE and oxalate (Fig. 3). The pellet obtained at high-speed centrifugation did contain PE and oxalate. No detectable amounts of proteins or carbohydrates were associated with this ultracentrifuged component. The control cells did not exhibit Al-citrate uptake nor Al-secretion properties. The heat-inactivated Al-stressed cells did not secrete Al upon incubation with citrate.

3.3. Characteristics of the Al extrusion system

This translocation of intracellular Al to the external environment was also observed albeit to varying degrees in the presence of such compounds as oxalate and D-glucose. Although isocitrate was as effective as citrate, the ability of other carbon sources to promote the secretion of the trivalent metal was comparatively slower. In the presence of oxalate, 90% of the Al was effluxed, while when succinate was utilized as the nutrient only, 22% of the intracellular Al was expelled to the external environment. When D-glucose was the substrate, the amount of Al secreted corresponded to only 5% of that initially localized in the cells. No detectable extrusion of Al was observed in the presence of EDTA or α-ketoglutarate (Table 1). However, when Al-citrate was the carbon source, more Al and PE were observed in the pellet obtained following centrifugation at high speed. To investigate the relationship between energy utilization and Al secretion, the susceptibility of the Al-loaded cells to secrete Al in the presence of DNP, DCCD and NaN_3 was investigated. These inhibitors did have only minimal negative influence on the extrusion of the metal. DNP, a dissipator of proton-motive force, DCCD, an inhibitor of ATP formation, and NaN_3, a compound known to interfere with the functioning of the respiratory chain, did not arrest Al secretion. Only a 10% inhibition was observed with DNP, while DCCD and NaN_3 did not appear to have any detectable negative impact on this process. The cells were also treated with cerulenin, an inhibitor of lipid biosynthesis, and their ability to extrude Al was monitored. A 43% diminution in the ability of the cells to

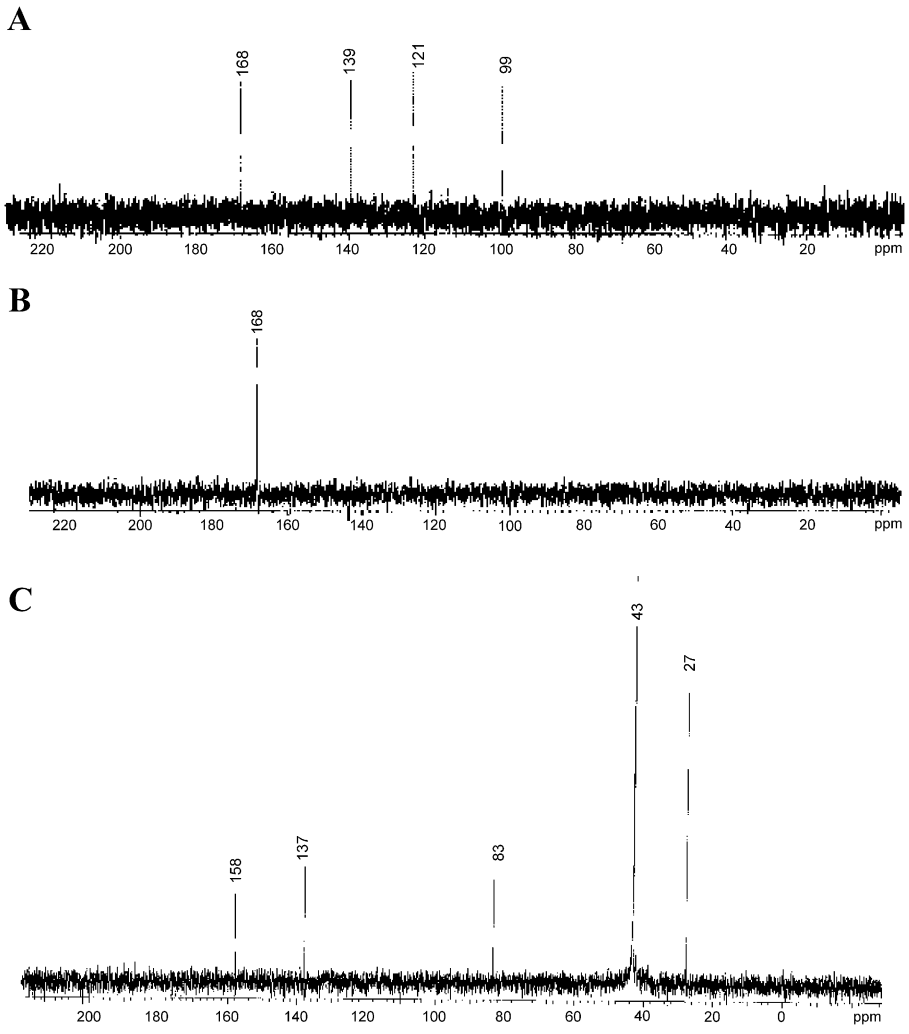


Fig. 1. Proton-decoupled ^{13}C NMR spectra obtained upon incubation of Al-citrate [2,4-$^{13}C_2$] with *P. fluorescens*. (A) Pellet obtained at 180,000 × *g* for 2 h. (B) Aqueous layer of pellet following $CHCl_3/CH_3OH/H_2O$ treatment. (C) Soluble cellular fraction.

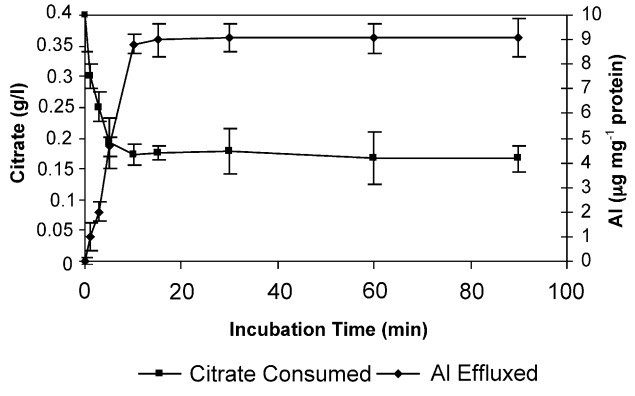


Fig. 2. Cells obtained at 40 h of growth in media supplemented with 15 mM Al-citrate were loaded with Al. Cells corresponding to 1.5 mg of soluble protein equivalent and 13.6 ± 1.2 μg Al were incubated with citrate at various time intervals. Citrate and Al were monitored as described in Materials and methods.

translocate the trivalent metal was observed (Table 2). However, cells heated at 60 °C were unable to efflux Al with either citrate or Al-citrate as substrate. When the reaction mixture was incubated at 4 °C, the extrusion of Al to the external medium diminished to 20% compared to that observed at 26 °C. The variation in pH did influence the capacity of the Al-loaded cells to secrete the trivalent metal. The secretion of Al was maximal at pH 6.0 and showed a marked decrease at higher pH. A 60% reduction in Al extrusion was observed at pH 8.2.

The foregoing data show that Al-loaded *P. fluorescens* secretes the trivalent metal upon incubation with a carbon source such as citrate, Al-citrate or oxalate. The Al is associated predominantly with PE and oxalate. The NMR data point to a pivotal role for oxalate in the detoxification of Al and reveal the transformation of citrate or Al-citrate to this dicarboxylic acid via the oxidation of glyoxylate. The occurrence of [2-$^{13}C_1$] in the glyoxylate and [CH_2-$^{13}C_1$] in the succinate moieties would indicate the cleavage of a product derived from the modification of [2,4-$^{13}C_2$] Al-

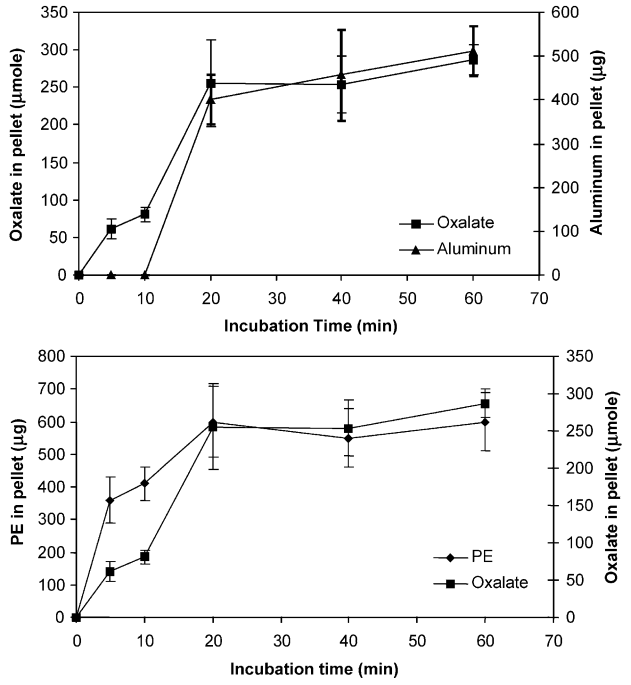


Fig. 3. Cells corresponding to 20 mg of protein with approximately 1.2 mg of Al^{3+} were incubated with 10 mM citrate in 8 ml of Tris buffer (pH 7.2). At timed intervals, following the removal of the bacterial cells, Al and oxalate content of the supernatant were monitored by the aluminon and oxalate oxidase assay. The supernatant fluid was further centrifuged at 180,000 × *g* for 2 h and the pellet was analyzed for Al, oxalate and PE as described in Materials and methods. Values are the means of three experiments performed in duplicate.

citrate and/or citrate. The generation of isocitrate would lead to these products. It is interesting to note that if succinate would have been generated via the regular tricarboxylic acid cycle, the carboxylic group [$^{13}C_1$] would have been labeled [24]. The NMR spectra showing the oxalate peak and resonances attributable to PE in the pellet obtained at high-speed centrifugation clearly argue for the notion that these moieties play a pivotal role in the extrusion of Al. Indeed, the $CHCl_3$ extract contained the PE peaks while the resonance indicative of oxalate was associated with the aqueous layer. The amount of Al extruded is concomitant to citrate consumed. This Al secretion process does not appear to be either fuelled by a proton-motive force or by ATP hydrolysis as DNP and DCCD were ineffective as inhibitors. NaN_3 that interferes with oxidative phosphorylation did not affect Al extrusion either. This is in sharp contrast to numerous reports describing the detoxification of metals in various organisms as a consequence of ATP hydrolysis or chemiosmotic exchanger [4,25,26]. The present study implicates a mechanism for the removal of Al from the cell that does not appear to be directly fuelled by either ATP or a proton-motive force but is aided by cellular membrane component(s) and oxalate. The association of Al with PE, an important constituent of bacterial membranes, clearly indicates a pivotal role of latter in the extrusion process. Membrane vesicles have been shown to contribute to the resistance of microorganisms to antibiotics and in the translocation of metals, proteins and various biological molecules intracellularly [27,28].

The insensitivity of the export machinery to the inhibitors of the energy-generating system and the inhibitory influence of cerulenin, an inhibitor of lipid synthesis [14] on the secretory pathway mediating the extrusion of Al, clearly demonstrates that the membrane components play a pivotal role in this process and that energy utilization is not instrumental for the expulsion of the trivalent metal. Since citric acid or a carbon source is essential for the secretion of the trivalent metal, it is not unlikely that these moieties may be involved in synthesis of a component(s) involved in the processing of Al prior to its association with the membrane constituents for the efflux process and/or the genesis of the membrane component(s). The ^{13}C NMR data do indeed confirm this possibility. The inability of EDTA, oxaloacetate and α-ketroglutarate to effect the secretion of Al would indicate that either these moieties could not be metabolized or could not get access to the cells. The fact that EDTA did not help release Al from the microbe provides clear evidence that the Al is tightly associated with the cellular components and could not be competitively sequestered by the tetracarboxylic acid. The fact the heat-inactivated cells were unable to secrete Al in the presence of the tricarboxylic acids clearly points to the biological nature of this process.

Table 1
Effects of various substrates on Al secretion by *P. fluorescens*

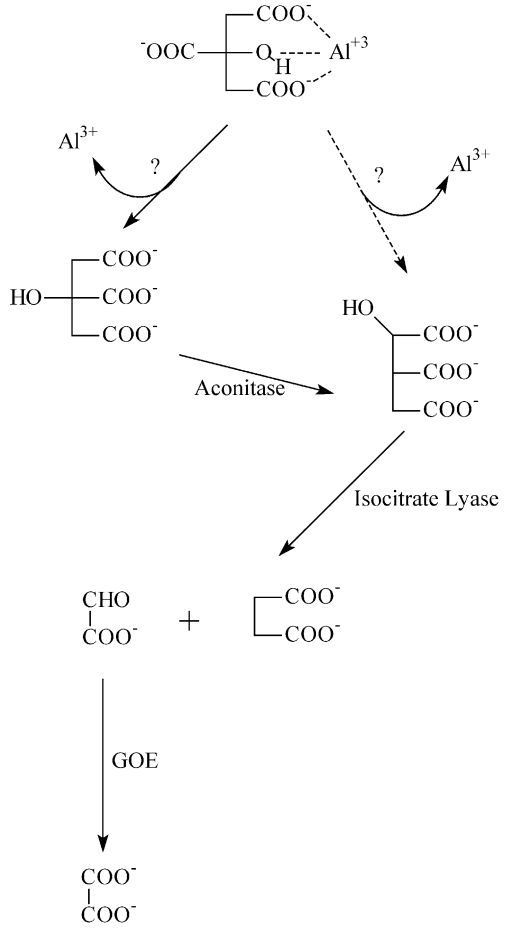
Substrate	Aluminum extruded (%)
Nil	0
Citric acid	100 ± 12.6
Isocitric acid	100 ± 10.6
Oxalic acid	92 ± 9.4
Aspartic acid	90 ± 7.2
Glucose	22 ± 3
Succinic acid	12 ± 2.6
Oxaloacetate	6 ± 3.1
Glycine	6 ± 2.8
α-Ketoglutaric acid	0 ± 0.98
EDTA	0 ± 1.2

Bacterial cells (1.5 mg soluble protein equivalent and 13.6 ± 1.2 μg of Al) were subjected to 10 mM of these substrates respectively for 1 h. Following the removal of the cells, Al was monitored. 100% represents 12.7 ± 1.6 μg of Al.

Table 2
Aluminum extrusion in *P. fluorescens* in the presence of different inhibitors

Inhibitor	Aluminum Extruded (%)
Nil	100±12.6
DNP	98±10.2
DCCD	91±8.9
$HgCl_2$	92±7.3
Nan_3	97±11.5
Cerulenin	57±4.6

Bacterial cells (1.5 mg soluble protein equivalent and 13.6±1.2 μg of aluminum) was treated with these inhibitors respectively. Following the removal of the cells, aluminum secreted in the supernatant was analyzed. 100% represents 12.7±1.6 μg of aluminum.



Acn : Aconitase
ICL : Isocitrate Lyase
GOE: Glyoxylate oxidizing enzyme

Scheme 1. A proposed model for the formation of oxalate from citrate in Al-stressed *P. fluorescens*.

Furthermore, control cells did neither affect the uptake nor the translocation of metal, thus demonstrating this characteristic was specific to the cells exposed to Al. The temperature dependence of this process would indicate that the proteins mediating the synthesis of the lipids and/or the moiety involved in the immobilization of Al prior to its extrusion and/or other unknown components of this secretory mechanism are inactivated at low or high temperature. The inhibition of Al efflux observed at higher pH would indicate that the molecules aiding the passage and/or the transformation of the trivalent metal to the external environment are sensitive to pH. The other possibility that Al precipitates in cells at higher pH may also be operative.

Thus, it is tempting to postulate that following the processing of the carbon sources into products that participate in the sequestration of Al and the formation of lipids, the trivalent metal is secreted into the external environment. This process does not appear to involve unregulated membrane blebbing, as neither lipopolysaccharide nor proteins were detected with these components. The severity of the stress and the limitation of cellular space would render such a strategy quite attractive. Indeed, cells adapted to Al stress have been shown to grow faster in subsequent Al media [11,29]. Simple efflux fuelled by ATP hydrolysis or chemiosmotic gradient would impose a significant burden on the cellular energy budget. Furthermore, this secretory vehicle may allow the extrusion of other metals and may not be governed by strict specificity requirement as in the case of an ATP-dependent pump. It is interesting to note that when the cells were exposed to Al in a medium with 100-fold less phosphate, the organism immobilized the trivalent metal as soluble metabolite [12].

The proposed Scheme 1 depicts an interesting model for the production of oxalate from Al-citrate or citrate and eventual secretion of Al. In conclusion, these data imply that *P. fluorescens* utilizes an energy-independent process to secrete Al from the cells. PE and oxalate are two important constituents of this secretory pathway. The dicarboxylic acid is obtained via the oxidation of glyoxylate, a product derived from the transformation of Al-citrate. This packaging and expulsion of Al in association with PE and oxalate is a novel mechanism that allows this organism to avert the challenge posed by the trivalent metal.

Acknowledgements

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