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Adaptation of *Pseudomonas fluorescens* to Al-Citrate: Involvement of Tricarboxylic Acid and Glyoxylate Cycle Enzymes and the Influence of Phosphate

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Abstract. The degradation of Aluminum-citrate by *Pseudomonas fluorescens* necessitated a major restructuring of the various enzymatic activities involved in the TCA and glyoxylate cycles. While a six-fold increase in fumarase (FUM EC 4.2.1.2) activity was observed in cells subjected to Al-citrate compared to control cells, citrate synthase (CS EC 4.1.3.7) activity experienced a two-fold increase. On the other hand, in the Al-stressed cells malate synthase (MS EC 4.1.3.2) activity underwent a five-fold decrease in activity. This modulation of enzymatic activities appeared to be evoked by Al stress, as the incubation of Al-stressed cells in control media led to the complete reversal of these enzymatic profiles. These observations were further confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy. No significant variations were observed in the activities of other glyoxylate and TCA cycle enzymes, like isocitrate lyase (ICL EC 4.1.3.1), malate dehydrogenase (MDH EC 1.1.1.37), and succinate dehydrogenase (SDH EC 1.3.99.1). This reconfiguration of the metabolic pathway appears to favour the production of a citrate-rich aluminophore that is involved in the sequestration of Al.

Aluminum is a trivalent metal that is not yet known to be essential for any biological activity. However, its toxicity has become a major cause of concern, as various abnormalities have been attributed to this metal. Its interference with Fe^{3+} metabolism, its mimicking of Ca^{2+}, a crucial secondary messenger, its interaction with membrane lipids, and its association with ATP are some of the reported molecular pathways via which Al exerts its toxic influence in various cellular systems [8, 16, 20]. Neurological disorders, retardation in root formation, and abnormal eggshell formation are some of the abnormalities triggered by Al. Hence, the occurrence of bioactive forms of Al in elevated amounts may pose a serious threat to the environment. The complexation of this metal with organic acids, such as citrate, succinate, and malate, may enhance the bioavailability of Al [7, 9].

Our laboratory has recently uncovered a pivotal role for oxalic acid in Al detoxification in *Pseudomonas fluorescens* exposed to Al-citrate. In this system, enhanced isocitrate lyase and glyoxylate oxidizing activities appeared to contribute to the production of oxalate. Subsequently, the Al in association with oxalate is extruded from the cells via a process aided by phosphatidylethanolamine (PE) [10, 11]. The level of phosphate in the medium (64 mM) appears to have a critical influence on this detoxification strategy. If the phosphate concentration is decreased 100-fold—from 64 mM to 0.64 mM—the organism elaborates a soluble metabolite that is involved in the sequestration of Al. In this instance, most of the trivalent metal is localized as a soluble organic moiety in the spent fluid [3]. As this microbe invokes disparate Al-detoxification strategies depending on the availability of phosphate, it is important to delineate the biochemical pathway operative in *Pseudomonas fluorescens* subjected to Al-citrate in a milieu with 0.64 mM phosphate. In this report, we describe how the microorganism modulates FUM, CS, and MS in an effort to avert the toxic challenge posed by Al. The biogenesis of a citrate-rich aluminophore is also discussed.

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Materials and Methods

All chemicals were reagent grade. AcetylCoA, serum albumin, 2,4-dinitrophenylhydrazine (DNPH), 5,5 dithio-bis-2-nitrobenzoic acid (DTNB), and nicotinamide adenine dinucleotide (NAD^+/NADH) were from Sigma (St. Louis, MO, USA). The Bradford assay kit was from Biorad. The microbial strain, *Pseudomonas fluorescens* ATCC 13525, was obtained from the American Type Culture Collection (Rockville, MD, USA) and was cultivated in a mineral medium, consisting of Na_2HPO_4 (0.06 g), KH_2PO_4 (0.03 g), NH_4Cl (0.8 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and citric acid (4.0 g), in 1 L of deionized distilled water. Aluminum chloride (15 mM) was complexed to citrate prior to sterilization. In the control cultures, citrate was the carbon source, while in the Al-stressed cultures, Al-citrate was the sole source of carbon. The pH was adjusted to 6.8 with dilute NaOH. This medium was also supplemented with trace elements as described in Anderson et al. [2]. Cellular growth was measured by monitoring solubilized proteins, following digestion with 1 M NaOH by the method of Bradford [4].

Preparation of cellular fractions and enzymatic analyses. Cells were recovered by centrifugation at 9,600 g for 15 min. The pellet, containing the cells, was washed once with 0.85% NaCl and subsequently with 50 mM Tris buffer (pH 7.3) containing 1 mM phenylmethylsulfonylfluoride (PMSF). The cells were resuspended in this buffer and disrupted by sonication. Unbroken cells were removed by centrifugation at 1,600 g. The supernatant was then spun at 181,000 g for 60 min in order to separate the soluble cellular components and membrane fraction. The membrane fraction contained in the pellet was resuspended in 10 mL Tris buffer, sonicated again, and spun at 181,000 g for 20 min. This step minimized cytoplasmic contamination in the membranes. The protein contents of both the membrane and soluble fractions were determined with the aid of the Bradford assay.

CS activity was determined in 25 mM Tris buffer (pH 7.3), using 5 mM oxaloacetate and 1 mM acetylCoA as substrates. The activity of this enzyme that was localized in the soluble reaction was monitored spectrophotometrically by measuring the formation of the sulfydryl in Coenzyme A with the aid of DTNB at 412 nm. Coenzyme A was utilized as the standard [18]. The activities of MDH, FUM, ICL, and MS were all measured by monitoring either the formation or the disappearance of ketoacids with the aid of DNPH at 450 nm [17]. All these enzyme assays were performed in 25 mM Tris buffer (pH 7.3) containing 5 mM $MgCl_2$. MDH activity was discerned by measuring oxaloacetate production with malate as substrate. Similarly, FUM activity was monitored in terms of the two-step reaction, resulting in the production of oxaloacetate with fumarate as substrate. For ICL activity, glyoxylate production from isocitrate (2 mM) was monitored. Glyoxylate was employed as the standard in order to quantitate this activity. The activity of malate synthase was measured in terms of glyoxylate utilization and Coenzyme A formation, respectively. The reaction mixture consisted of 0.2 mM glyoxylate and 0.2 mM acetyl-CoA. All these reactions were incubated for 30 min, and 0.2-mg equivalent of either soluble or membrane protein were utilized. SDH was quantitated by the reduction of dichloroindophenol (DCPIP) at 550 nm. The reaction mixture contained 5 mM KCN, 10 mM succinate, and 0.2 mg/mL^{-1} of protein equivalent of the membrane fraction [14].

Modulation of enzymatic activities. *Pseudomonas fluorescens* was grown at various time intervals and the cellular fractions were analyzed for enzymatic activities. To evaluate the influence of Al on this process, the cells were harvested from Al media at time intervals when there were the most variations in the enzymatic activities between the control and Al cultures. Al-stressed cells (corresponding to 2 mg of protein) were transferred to the control media (without added Al) and allowed to incubate for 4 h. These cells were subsequently harvested and resuspended in fresh Al media for 4 h. Following the fractionation of these cells, the enzymatic activities were monitored as described before.

Isolation and partial characterization of the aluminophore. Following the attainment of the stationary phase of growth, the spent fluid was centrifuged at 10,000 g in order to afford a bacterial pellet and a supernatant. Most of the aluminum was localized in the latter fraction. The supernatant was lyophilized and subjected to a Biogel-P2 column that was equilibrated with double-distilled H_2O. The Al-rich fractions were pooled and were further analyzed for Al and citrate, before and after hydrolysis with dilute HCl [13, 15]. ^{13}CNMR spectra for the metabolite was obtained on a Bruker-ARX 400 NMR spectrometer. A 5-mm inverse geometry triple probe (^{1}H, ^{13}C, ^{15}N) was used, for which the ^{1}H 90° pulse was 11 μs and the ^{13}C 90° pulse was 14 μs. ^{13}C multiplicities were determined using a standard Bruker DEPT pulse sequence; DEPT 135 where CH_3 and CH appear positively phased and CH_2 is negatively phased.

To further confirm the biogenesis of this aluminophore, *Pseudomonas fluorescens* was grown in labeled Al-citrate [2,4-$^{13}C_2$] (1:1) and the supernatant was analyzed at 30 h of growth using a Varian Gemini 2000 spectrometer. The conditions were same as described in [11].

^{1}H NMR studies of enzymatic activities from the cellular fractions. The reaction mixture (1 mL) contained 500 mg mL^{-1} of either soluble or membrane protein and the respective substrate (1 mM), in 10 mM phosphate buffer pH 7.2. In the case of the FUM and MDH that were localized in the membrane fraction, fumarate and malate were used as substrates, respectively. CS and MS were associated with the soluble components and were probed, with oxaloacetate plus acetylCoA and glyoxylate plus acetylCoA as substrates, respectively. After 10–30 min of incubation, the samples were freeze dried and reconstituted in D_2O. Spectra were obtained on a Varian Gemini 2000 spectrometer. The use of the homodecoupler allowed for the suppression of H_2O. The parameters were adjusted as follows: continuous modulator; delay, 0; pulse, 2; acquisition time, 1; pulse width, 90°; decoupler, low power to 2000; and the number of transients, 5 (subsequently changed to 200 following the suppression of H_2O).

Results and Discussion

In a medium with 0.64 mM phosphate, Al is immobilized in the supernatant, in soluble form [3]. Biogel-P_2 chromatography yielded a sharp peak that was rich in Al. The proton-decoupled spectrum of this metabolite showed numerous peaks in the 45–41 ppm, the 75–73 ppm, and the 176–182 ppm regions (Fig. 1). Only the peaks at 45–41 ppm were shown to be CH_2, while the other peaks were not directly bound to an H-atom. The peaks at 176–182 ppms are attributed to carboxylic and ester groups. Upon acid hydrolysis, citrate was found to be an important component of this metabolite. Furthermore, the supernatant of the Al-citrate [2,4-$^{13}C_2$] (1:1) labeled cultures showed six peaks at 41–45 ppm that were indicative of CH_2 groups. (Note at time 0, the culture medium prior to microbial growth gave a singular peak at 42–43 ppm.)

The activity of CS, FUM, and MS showed marked variations in the control and Al-stressed cells. CS, an enzyme that condenses oxaloacetate and acetylCoA to

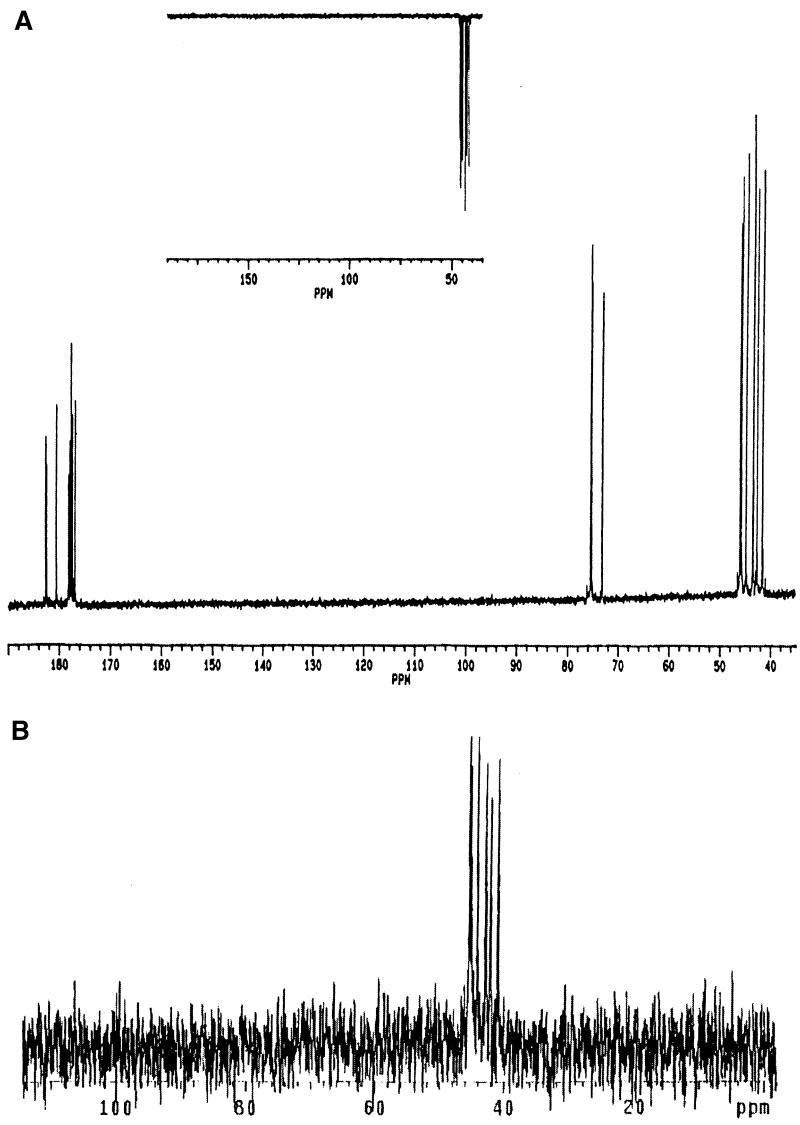

Fig. 1. **(A)** ^{13}C NMR of soluble aluminum metabolite (aluminophore) obtained during the stationary phase of growth, following a Biogel-P_2 chromatography. **(B)** ^{13}C NMR of supernatant obtained from cultures of *Pseudomonas fluorescens* grown in labeled Al-citrate (2,4-$^{13}C_2$) for 30 h.

citrate, experienced a two-fold increase in the Al-stressed cells, compared to the control cells. While there was a sixfold increase in FUM activity in the Al-stressed cells, the MS activity was found to be five-fold lower in the Al-stressed cells than in the controls (Fig. 2). Activities of MDH, ME, ICL, and SDH did not show any appreciable differences in the control and in the Al-stressed cells (Table 1). The changes in enzymatic activities were also followed by ^{1}HNMR spectroscopy. The peak at 6.2 ppm, indicative of double bond in fumarate, disappeared quickly with the concomitant formation of a peak at 2.2 ppm, attributable to the CH_2 of oxaloacetate in membranes obtained from Al-stressed cells (Fig. 3). No such change was observed with control cells. Similarly, the CH_3 peak (1.3 ppm) from acetylCoA did not change upon incubation of the soluble fraction from the Al-stressed cells with glyoxylate. However, the soluble fraction of the control cells readily consumed the CH_3 peak upon the incubation of acetylCoA and glyoxylate (data not shown). In order to further elucidate the influence of Al on these various metabolic enzymes, cells were subjected to the Al-cultures and transferred to fresh control medium for 4–8 h. A marked decline in activities of CS, and FUM was observed, while the MS activity saw an increase. However, when these cells were subsequently exposed to fresh Al media, a reverse trend was recorded. These observations clearly point to a pivotal for Al as a modulator for these enzymatic activities (data for FUM shown, Fig. 4).

The aforementioned results demonstrate that in order to survive elevated levels of Al in a medium with 0.64 mM phosphate, various metabolic enzymes in

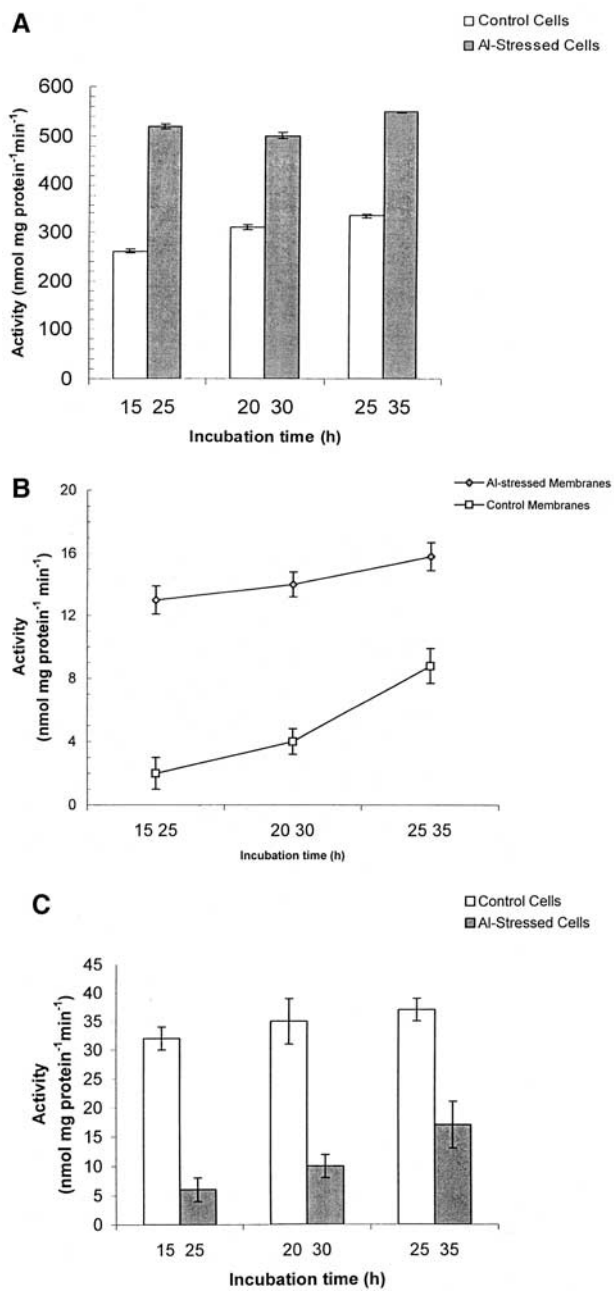

Fig. 2. **(A)** Citrate synthase activity in the cell-free extract (soluble fraction) from control and Al-stressed *P. fluorescens,* obtained at various time intervals that correspond to the same growth phases ($n = 4$; values are ± SE). **(B)** Fumarase activity in control and Al-stressed *P. fluorescens,* obtained at various time intervals that correspond to similar growth phases. The fumarase enzyme was localized in the membrane fraction, the activity measured in terms of oxaloacetate produced ($n = 4$; values are mean ± SE). **(C)** Malate synthase activity in control and Al-stressed cells, at various time intervals that correspond to similar growth phases. The utilization of glyoxylate was monitored ($n = 3$; values are mean SE).

Pseudomonas fluorescens were either upregulated or downregulated. In this instance, CS and FUM activities were increased, while the activity of MS experienced a significant decrease. This is in sharp contrast to changes observed when the same microbe was grown in an Al medium with 100-fold more phosphate. A sharp rise in ICL activity appeared to drive the adaptation of this organism to Al, as oxalate is a key participant in this process. Hence, phosphate seems to be a critical ingredient that dictates the detoxification strategies invoked in order to nullify Al in the *Pseudomonas fluorescens* [10, 11, 12].

In this study, the ^{13}CNMR and hydrolytic studies point to the involvement of a citrate derivative in the detoxification of Al. This aluminophore appears to be rich both in carboxylic groups and in ester linkages. The requirement for this precursor may indeed be fulfilled by the increase of CS activity. Indeed a two-fold increase in the activity of this enzyme was observed. FUM, a key enzyme that participates in the generation of oxaloacetate, an important substrate for the production of citrate, did undergo a six-fold increase. The other metabolite needed for this condensation reaction of oxaloacetate to citrate, is acetylCoA. To assure an adequate availability of this moiety, the activity of MS appeared to be drastically curtailed. This enzyme reacts with acetylCoA and glyoxylate to produce malate. Its inhibition will allow the funneling of essential acetyl CoA towards CS, and eventually, to the production of citrate. FUM, on the other hand, will lead to increased production of malate and eventually oxaloacetate. Thus, an increase in FUM and CS activities and a decrease of MS will result in an enhanced production of citrate, a metabolite pivotal in the genesis of this aluminophore. The increase in CS activity as a consequence of Al has been observed in plants. Plants harboring gene(s) with increased expression of CS have been shown to proliferate in acidic environment [6]. It is also interesting to note that ICL, which plays a key role in anabolic reactions, especially when either citrate or acetate is the only source of carbon, did not experience any change in activity [5]. However, MS, an enzyme that works in tandem with ICL as part of the glyoxylate cycle, appeared to be decoupled. This will allow the generation of energy via the formation of succinate and also the production of the aluminophore by diverting the essential acetylCoA towards CS. It is important to note the activities of these enzymes fluctuated as growth progressed. Thus, it is likely that these enzymes are modulated depending on the necessity of the cells to detoxify the toxic Al species. As Al is immobilized, the activities of these enzymes return to their normal levels. The modulation of these activities appeared to be a direct consequence of Al stress. When

Table 1. Activities of various enzymes from control and Al-stressed cultures tested in the early stationary phase of growth

Enzyme	Control Culture (nmol/mg protein^{-1}/min^{-1})	Al-stressed cultures (nmol/mg protein^{-1}/min^{-1})	Control fractions + Al-citrate (nmol/mg protein^{-1}/min^{-1})	Control fractions + $AlCl_3$ (nmol/mg protein^{-1}/min^{-1})
Citrate synthase	260 ± 4	530 ± 3	269 ± 3	245 ± 4
Malate synthase	32 ± 0.2	6 ± 0.2	30 ± 0.5	38 ± 0.5
Fumarase	2 ± 0.1	13 ± 0.1	2 ± 0.1	2 ± 0.1
Malate dehydrogenase	8 ± 0.2	10 ± 0.2	10 ± 0.2	11 ± 0.3
Isocitrate lyase	18 ± 2	24 ± 3	18 ± 1	18 ± 2
Succinate dehydrogenase	70 ± 10	65 ± 6	74 ± 11	78 ± 12

the Al-exposed cells with maximal FUM and CS activities were transferred to control cultures, the activities of these enzymes fell to the normal levels. A similar observation was recorded with MS. However, in the latter instance, a marked increase of activities was observed when the Al-stressed cells were transferred to control media. These data point to the elevation of the activities in FUM and CS and the diminution in MS, which may be due to the cells responding to Al rather to the metal interacting directly with the enzymes. Indeed, no significant variation in enzymatic activities was observed upon the incubation of these enzymes with either $AlCl_3$ or Al-citrate. When the microbe was grown in an Al medium with 100-fold more phosphate, an entirely disparate detoxification strategy was observed [10, 11]. Thus, it is very likely that the concentration of phosphate is dictating the detoxification process. As the level of phosphate is higher in the latter instance, phosphatidyethanolamine (PE) is utilized in the immobilization process. It appears that the organism switches to a stratagem that invokes

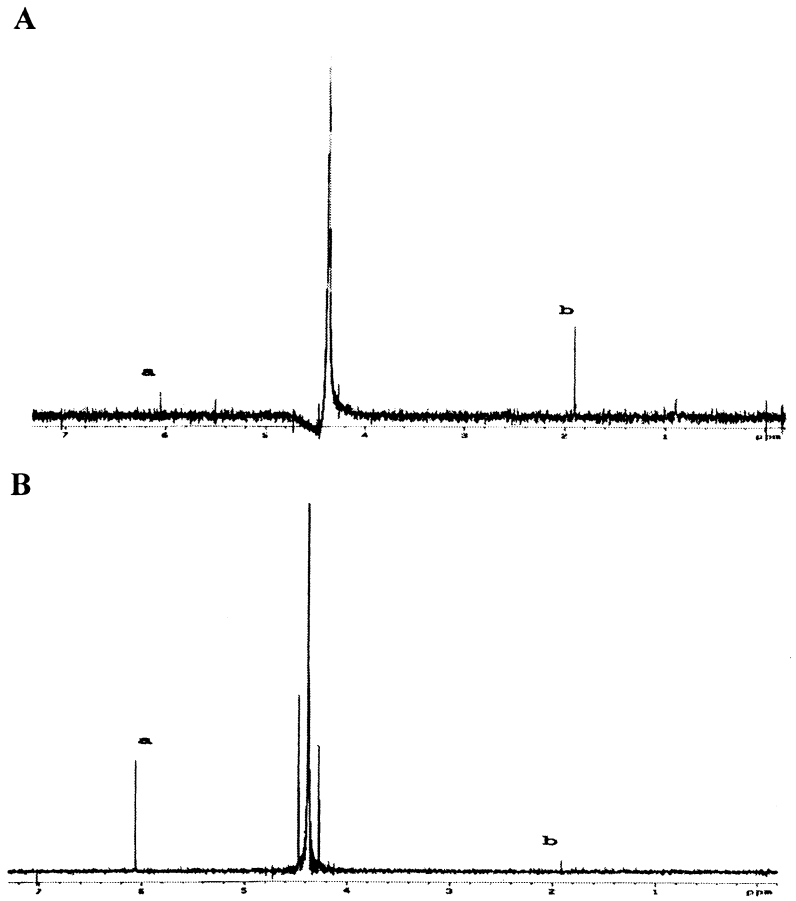

Fig. 3. ^{1}H NMR spectrum obtained upon incubation of membrane components with fumarate (1 mM): **(A)** membranes from Al-stressed cells, **(B)** membranes from control cells. Note the disappearance of the fumarate peak **(a)** in the Al-stressed membranes (**a** = 6.1 ppm; **b** = 1.9 ppm).

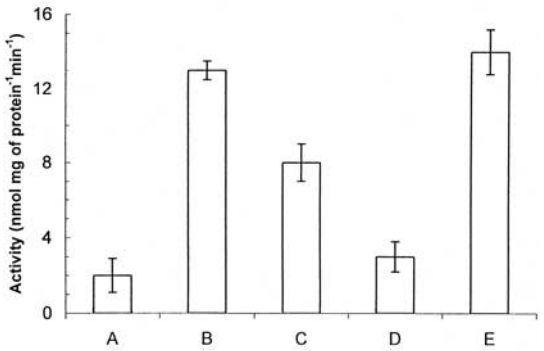

Fig. 4. Modulation of fumarase activity by Al: **(A)** cells grown 15 h in control medium; **(B)** cells grown 25 h in Al-stressed medium; **(C, D)** 2 mg of soluble protein equivalent of cells from **B,** incubated 4 h and 8 h respectively, in fresh control medium; **(E)** cells from **D** were incubated 4 h in fresh Al-citrate medium (n = 3; values are mean ± SE).

the use of a polycarboxylated moiety to sequester the trivalent metal as the level of phosphate is decreased. This situation evoked the configuration of a unique pathway designed to generate citrate, an essential ingredient in the formation of the aluminophore. The enhanced production of oxaloacetate via FUM and the diversion of acetylCoA towards CS affected by the repression of MS appear to result in the eventual survival of the organism in the Al medium. The production of lipid by the enhanced activity of ME in fungi [19], and G6PDH in higher organisms [1], has been shown. These NADPH-generating systems trigger the synthesis of lipids, as NADPH is critical for the condensation of acetylCoA. Thus, in a complex metabolic pathway, a change in activities of only a few enzymes may increase or decrease the formation of a product. Figure 5 depicts a possible metabolic pathway that may lead to the production of the aluminophore in *Pseudomonas fluorescens.*

In conclusion, we have demonstrated that the cellular metabolism is reconfigured in an effort to avert the danger posed by Al. In this instance, the various biochemical pathways are designed to channel oxaloacetate and acetylCoA towards citrate, a key metabolite in the biogenesis of the aluminophore. Thus, the modulation of FUM increases oxaloacetate, while the regulation of MS assures sufficient supply of acetylCoA for citrate production via the enhanced activity of CS. This metabolic network allows the cells to respond to the dual stress imposed by Al and phosphate.

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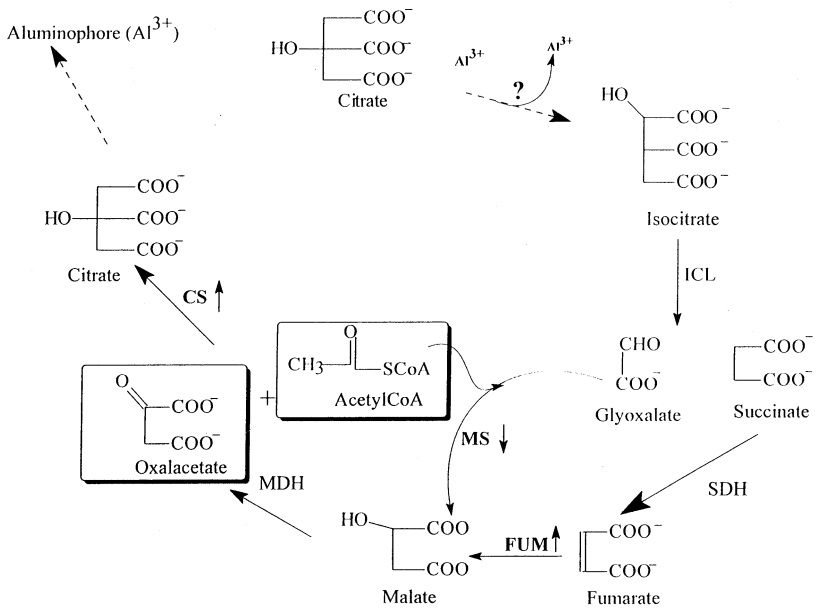

Fig. 5. A possible metabolic pathway leading to the production of an aluminophore in *Pseudomonas fluorescens.* (Note that the increased pools of oxaloacetate (FUM) and acetylCoA (MS) are channeled towards CS.

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